

**HYPOCHONDROPLASIA:**  
**CLINICAL AND MOLECULAR SPECTRUM AND**  
**RESPONSE TO GROWTH HORMONE THERAPY**

**MD THESIS SUBMITTED TO**  
**THE UNIVERSITY OF LONDON**

**DR. UMA RAMASWAMI**  
**LONDON CENTRE FOR PAEDIATRIC ENDOCRINOLOGY**  
**MIDDLESEX HOSPITAL, MORTIMER STREET**  
**LONDON**

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## **2. ABSTRACT**

Hypochondroplasia (HCH) is a genetic disorder of short stature with a wide spectrum of clinical severity, from short-limbed dwarfism to proportionately short children with diminution of the pubertal growth spurt. The invariable radiological feature is a lack of increase in interpedicular distance between lumbar vertebrae L1 to L5 and with short pedicles. 73 children with clinical and radiologically proven HCH were screened for the C1620A and C1620G mutations in the fibroblast growth factor receptor 3 (FGFR3) gene. Both these mutations resulted in an asparagine to lysine substitution in codon 540 (Asn540Lys) of the proximal tyrosine kinase domain of FGFR3. In mutation negative patients (n=45), single strand conformation polymorphism analysis was performed to screen for sequence variants in the tyrosine kinase and transmembrane domains of FGFR3 gene.

28/73 (38%) patients were heterozygous for the C1620A mutation and these patients had severe HCH with disproportionate short stature. No patient in this study had the C1620G mutations or mutations in the transmembrane domain described in Achondroplasia (ACH). The mutation negative patients although short, were not obviously disproportionate and presented later with short stature relative to their family height. A sequence variant, a nucleotide insertion in intron 12 was found in a small proportion of mutation negative patients and in normal individuals, presumably reflecting a sequence polymorphism with no functional significance.

The patients were divided into C1620A mutation positive (group 1) and C1620A mutation negative groups (Group 2) and the responses to

recombinant human growth hormone (r-hGH) therapy was analysed in both groups. The majority were prepubertal at the start of treatment (88%). 16 patients in Group 1 and 22 patients in Group 2 received r-hGH at a median dose of 30U/m<sup>2</sup>/week (16-44 U/m<sup>2</sup>/week). Responses to r-hGH therapy between 1-5 years were significant in both groups, with children under 10 years of age having a significantly better response. This response was predominantly due to an increase in the length of the back in the mutation positive group, thus accentuating the existing disproportion. In the mutation negative group, there was a more proportionate growth response. The response to growth hormone therapy in ACH was also analysed and compared with responses to r-hGH therapy in HCH. The C1620A positive HCH group had a similar response to r-hGH therapy when compared to ACH. The phenotype within these two groups was also similar. However, the severity of short stature and disproportion in children with C1620A positive HCH was less severe than those with ACH.

Genotyping of patients with HCH permits the definition of patients into C1620A positive and negative groups. This allows critical examination of the efficacy of growth hormone treatment of what is otherwise a rather heterogeneous group of patients, defined by radiological parameters alone. Further analysis of the 2.5kb mRNA performed in other centres has not identified any further mutations in FGFR3 in the C1620A mutation negative group. Future collaborative work with other centres will be needed to clarify the situation in these patients and whether they have mutations in FGFR3 or in other genes.

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### **5. ABBREVIATIONS**

Achondroplasia	ACH
Amplification Refractory Mutation System	ARMS
Analysis of variance	ANOVA
Asparagine to Lysine Substitution at codon 540	Asn540Lys
Base pair	bp
Deoxynucleosides triphosphates	dNTPs
Distilled Water	dd H <sub>2</sub> O
Ethylenediaminetetra-acetate	EDTA
Fibroblast Growth Factor Receptor 1	FGFR1

Fibroblast Growth Factor Receptor 2	FGFR2
Fibroblast Growth Factor Receptor 3	FGFR3
Fibroblast Growth Factor Receptor 4	FGFR4
Fibroblast Growth Factor	FGF
Glycine to Arginine substitution at codon 380	Gly380Arg
Growth Hormone	GH
Height	Ht
Hypochondroplasia	HCH
Magnesium Chloride	MgCl <sub>2</sub>
Minutes	min
Polymerase Chain Reaction	PCR
Potassium Chloride	KCl
Recombinant human growth hormone	r-hGH
Seconds	Secs
Single Strand Conformational Polymorphism	SSCP
Sitting Height	SH
Sodium Chloride	NaCl
Sodium Dodecyl Sulphate	SDS
Sodium hydroxide	NaOH
Standard deviation scores	SDS
Subischial leg length	SILL
Tetramethylammonium Chloride	TMAC
Tyrosine Kinase Domain	TKD

## **6. AIMS AND OBJECTIVES**

### **AIM**

This is a study of Hypochondroplasia – a genetic cause of disproportionate short stature. The aim of this study was to:

- Define the Hypochondroplasia (HCH) phenotype with respect to auxological and radiological criteria
- To screen for common mutations in the Fibroblast Growth Factor Receptor 3 gene (FGFR3) and to evaluate the role of mutation analysis in the postnatal diagnosis of Hypochondroplasia
- To establish the prevalence of these common mutations in our cohort of children.
- To analyse the response to recombinant human growth hormone therapy in children with Hypochondroplasia and Achondroplasia (ACH)
- To determine the relationship between genotype and phenotype and whether this relationship can be used to identify patients who will benefit from growth hormone therapy.

### **OBJECTIVES**

- Recruit cases for this study from paediatric growth clinics at the Middlesex Hospital and Great Ormond Street Childrens Hospital in London. To collect sequential, longitudinal growth data pre treatment and during treatment with recombinant human growth

hormone (r-hGH). To collect this growth and other data from documentation in growth cards and the clinical notes. Accredited measurers in the growth clinics will be responsible for collection of auxological data.

- Develop assays for detection of defined FGFR3 mutations (C1620A and C1620G) in Hypochondroplasia.
- To screen the Tyrosine Kinase Domain and Transmembrane Domain of FGFR3 in mutation negative patients, by Single Stranded Conformational Analysis (SSCP) in the first instance, with subsequent sequencing of SSCP variants.
- Establish and maintain a clinical database of all children in this study. This will include details of diagnosis, genotyping data, auxological parameters including height, sitting height, subischial leg length, height velocity, calculation of standard deviation scores of the above, onset of r-hGH therapy, dose and side effects.
- Information from this database will be used to analyse the response to growth hormone therapy, genotype-phenotype correlations, to assess the value of mutation analyses with respect to r-hGH response in common mutation positive groups and others. In addition, r-hGH treatment in ACH and HCH will also be compared.

## **7. INTRODUCTION**

### **7.1 Classification Of Skeletal Dysplasias**

The human chondrodysplasias are a genetically and phenotypically heterogeneous group of disorders. They result in developmental abnormalities in chondro-osseous tissue and the majority affects linear bone growth. The International Classification of Osteochondrodysplasias (1992) is currently based on the characteristic radiological features of this group of disorders. The variability in clinical criteria and also in the natural history of skeletal dysplasias, led to inconsistencies in classification which resulted in diagnostic inaccuracies (Aleck *et al.*, 1987). Phenotypic classification was therefore, not included in the revised classification in 1992. The new International Classification Of Osteochondrodysplasias (1992) includes information on mode of inheritance, the gene localisation and the defective proteins where available.

Chondrodysplasias are classified into three major groups: defects in tubular/flat bone and axial skeleton; disorganised development of cartilage and fibrous tissue (enchondromatosis and fibrous dysplasia) and idiopathic osteolysis. The major groups are further subdivided and then sub grouped to include over 150 types of Osteochondrodysplasias.



Skeletal dysplasias are inherited in an autosomal dominant fashion with 100% penetrance, although, the majority are due to *de novo* mutations (Murdoch *et al.*, 1970; McKusick *et al.*, 1973; McKusick 1978; McKusick 1997). The sporadic nature of the disease and the lack of a large number of familial cases for linkage analysis made molecular characterisation of chondrodysplasias difficult. However several genetic loci for the commoner skeletal dysplasias have now been identified (Horton 1997a) and surprisingly, for a complex process such as skeletal development, the number of genes involved is relatively few (Horton 1996 a; Horton 1997 a; Horton 1997 b).

## **7.2 Skeletal Development Of Long Bones**

The skeleton is formed from connective tissues and shares their mesodermal origin with muscle and vascular tissue. Local mediators of bone growth include insulin-like growth factor II, acidic and basic fibroblast growth factors, platelet derived growth factors and bone morphogenetic proteins. Other mediators of bone growth include interleukins, tumour necrosis factors, interferons, colony stimulating factors and prostaglandins. During osteogenesis, cartilaginous bone is enveloped by highly vascularised connective tissue. This is known as the perichondrium and consists of two layers, an inner layer with collagen filament secreting chondroblasts and an outer tougher fibrogenic layer. Chondrocytes within the inner layer of the perichondrium have the potential to divide and secrete collagen. The

chondrocytes facilitate the process of interstitial bone growth whereby, there is cartilage expansion with increase in bone length and diameter. During the process of remodeling and bone growth, the cartilaginous tissue is replaced by bony tissue and ossification centres are formed. Subsequently, bone mineralisation follows with deposition of predominantly carbonate and phosphate salts of calcium. The bone that is initially formed at the ossification centre is trabeculated, and eventually replaced by cortical bone. Despite the continuing process of cartilage being replaced by bone, as ossification occurs, cartilaginous growth is mainly responsible for the increasing length of long bones (Kember 1978; Sisson 1971).

Bone growth and maturation is a continuous process with bony destruction by osteoclasts and subsequent new bone formation by osteoblastic activity. The cartilaginous growth plate that enables bone length increase, is itself held in place and protected from damage due to shearing forces by the perichondrium that surrounds it. The chondrocytes within the epiphyseal growth plate are organised within layers depending on their stage of maturation. The four zones within the growth plates are the germinal, proliferative, hypertrophic and calcification zones. The cells within the germinal layer are the progenitor or stem cells. The matrix in this layer has type II collagen fibrils that inhibit calcification and this zone is highly vascularised (Kember 1960). The chondrocytes within the proliferative zone are organised in columns and the majority of cell replication occurs in this zone with subsequent clonal expansion of chondrocytes. The width of

the growth plate reflects the rate at which cell division occurs and as a result the rate of longitudinal bone growth. Within the hypertrophic zone, the amount of type II collagen fibril secretion is reduced and is replaced by type X collagen production, which is specific for precalcifying and calcifying cartilage. The cells in this zone stop dividing, mature and have a high level of mineralisation, with increasing levels of alkaline phosphatase. Finally, in the calcification zone of the growth plate, there is poor vascularisation and increase in matrix calcification. The rate of ossification eventually overtakes cartilage formation and is restricted to cartilage present in articular surfaces and the cartilaginous growth plates being replaced by bone only after the adolescent growth spurt. The cells subsequently degenerate and are incorporated into metaphyseal bone (Ohlsson *et al.*, 1993; Sisson 1971; Kember 1978).

Enchondral ossification is the process by which cartilage is converted to bone. It has been shown in Achondroplasia (ACH), the commonest genetic form of short stature in man, that there is defective enchondral ossification of the growth plates of long bones. During development, bone initially forms at the ossification centres within the diaphysis and subsequently the growth plate is differentiated at either end. After the pubertal growth spurt, the growth plate becomes thinner and is eventually resorbed and bone formation is completed. This process of closure of growth plates is not uniform and occurs at different time scales within the skeleton. Although several hormones including thyroid (Thorngren *et al.*, 1973; Lewinson *et al.*,

1989) and sex hormones (Frantz *et al.*, 1965; Bourguignon 1988) are needed for normal bone growth, growth hormone has a dominant effect in longitudinal bone growth (Ohlsson *et al.*, 1992; Ohlsson *et al.*, 1993).

A good understanding of the pathogenic mechanisms in Osteochondrodysplasias is important and might improve the classification of skeletal dysplasias and aid in diagnosis, prognosis and genetic counseling. It would also contribute to the understanding of normal enchondral growth. The pathogenesis of skeletal dysplasias was studied by Stanescu and colleagues (Stanescu *et al.*, 1984; Stanescu *et al.*, 1982a; Stanescu *et al.*, 1982b; Maroteaux *et al.*, 1980). Histochemical, immunohistochemical, electron-microscopic, and microchemical studies on cartilage growth plates revealed specific abnormalities suggestive of a specific biochemical defect. In pseudoachondroplasia, non-collagenous protein accumulated in the rough endoplasmic reticulum of chondrocytes and a proteoglycan species that normally is present in the extracellular matrix, is not seen. The accumulated material was stained with antibodies against the core protein of proteoglycan. This showed that the abnormal core protein of proteoglycan species was not properly transferred to the Golgi system (Stanescu *et al.*, 1982a; Stanescu *et al.*, 1982b). In Kniest syndrome, intracytoplasmic accumulation of metachromatic material, dilatation of rough endoplasmic reticulum, and an abnormal gel-electrophoretic pattern of cartilage proteoglycans suggested an abnormality of cartilage proteoglycan metabolism. Abnormalities related to degradative lysosomal processes of

proteoglycans in chondrocytes were found in spondylometaphyseal dysplasia (Stanescu *et al.*, 1984). An abnormal organization of type-II collagen was found in fibrochondrogenesis. In diastrophic dysplasia, an abnormal organization of collagen was found in areas of inter territorial matrix, around many degenerated cells and in the lacunae of cells without ultra structural signs of degeneration (Stanescu *et al.* 1982b).

The histological and biochemical changes in growth cartilage of homozygous ACH complemented similar studies of underlying cartilaginous defects in heterozygous ACH (McKusick 1978). Homozygous achondroplasia is a rare and severe form of achondroplasia, which affects 25% of children born to heterozygous ACH parents. The homozygous variant is usually lethal but they have occasionally survived beyond infancy (Hall *et al.*, 1969 b; Pauli *et al.*, 1983). Prenatal diagnosis is possible in this condition (Bellus *et al.*; 1994). Severe defects of growth cartilage of long bones, absence of regular column formation, minimal proliferation and hypertrophy of cartilage growth cells, abnormal vasculature and irregularly arranged hypertrophic cells interspersed with resting cartilage and thin epiphyseal lines have been described in homozygous achondroplasia (Aterman *et al.*, 1983). Similar studies in heterozygous ACH have shown abnormal collagen organisation (Stanescu *et al.*, 1970; Stanescu *et al.* 1972; Ponseti *et al.* 1970; Maynard *et al.*, 1981). These studies showed that apart from the growth plate cartilage in tibia and femur that were abnormal, the epiphyseal cartilage, vertebral growth plates and the ileac crest growth plate

were in fact morphologically normal in appearance. In summary, there are several differences in the development of the skeleton in ACH and are as follows: normal vertebral body height due to the normal vertebral and iliac crest growth plate cartilage. The sacrum and vertebral pedicles are short as these usually grow during enchondral ossification and this is known to be defective in ACH. The interpedicular distance is narrow due to the shortening of the sacrum (Langer *et al.*, 1967). Anterior wedging of the upper lumbar vertebrae results in thoracolumbar kyphosis.

It was postulated that in ACH there might be abnormalities of a growth factor or its receptor, resulting in a specific defect in cell proliferation in the growth plate cartilage (Stanescu *et al.*, 1990). Recent studies have shown that mutations in fibroblast growth factor receptor 3 (FGFR3) gene disrupt enchondral ossification of bone and will be discussed in more detail later.

### 7.3 Achondroplasia (ACH) and ACH Like Diseases

These diseases are characterised by abnormalities in the long bones of the skeleton, which include disproportionate short stature of varying severity. At the severe end of the spectrum is Achondroplasia (ACH) and Thanatophoric Dysplasia (TD). At the milder end, with a varied clinical spectrum, is Hypochondroplasia (HCH). Those with a severe phenotype resemble ACH and the milder ones present with varying degrees of short stature and delayed puberty.

The term achondroplasia meaning total absence of cartilage was first used by Parrot in 1878. The incidence of achondroplasia is 1 in 26,000 and it is inherited as an autosomal dominant trait with 100% penetrance, although 80% of cases are sporadic (Francomano *et al.*, 1988; Murdoch *et al.*, 1970; Oberklaid *et al.*, 1979). Increasing paternal age at the time of conception has been observed to be associated with an increasing incidence of ACH, suggesting de novo mutations of parental origin (Thompson *et al.*, 1986; Penrose 1955). Homozygous ACH rarely survive beyond infancy although there are reports of a few cases surviving into childhood (Pauli *et al.*, 1983).

Achondroplasia is characterised by rhizomelic dwarfism, macrocephaly, bulging forehead, marked lumbar lordosis, genu varum, trident hands and hyperextensibility of most joints (Dawson *et al.*, 1980; Mckusick *et al.*,

1973; McKusick 1978; Maroteaux *et al.*, 1964; Oberklaid *et al.*, 1979; Scott Jr *et al.*, 1976). The flat nasal bridge and midface hypoplasia is a result of the defective growth of the skull and face (Ponsetti *et al.*, 1970).

The radiographic findings include a short base of skull, a small foramen magnum, narrowing of the entire length of the spinal canal with a decrease in vertebral bodies dimensions and squared iliac crests with a narrow sciatic notch (Oberklaid *et al.*, 1979; Hecht *et al.*, 1986; Hecht *et al.*, 1989 a; Hecht *et al.*, 1989 b). The long bones have flared metaphyses and the metacarpals are of the same length, which gives the characteristic "trident" hand (Wynne-Davies *et al.*, 1981; Ponsetti *et al.*, 1970). The iliac crest growth plates in children with achondroplasia were noted to be shorter than that of age matched controls (Horton *et al.*, 1978; Horton *et al.*, 1977). Defective chondrocyte proliferation and enchondral bone formation as discussed previously, result in disruption of bone development in achondroplasia (Ponsetti ., 1970; Horton *et al.*, 1978; Stanescu *et al* 1984; Stanescu *et al.*, 1990), with the intramembranous and periosteal ossification appearing normal.

Thoraco lumbar kyphosis due to anterior wedging of the upper lumbar vertebrae is associated with increased neurological complications (Cohen *et al.*, 1967; Hall *et al.*, 1988). An abnormal posture during early infancy with inadequate support to the back, associated with muscular hypotonia, is said to worsen the kyphosis. Computerized tomographic dimensions of the



foramen magnum of achondroplastic individuals were compared to standards established for normal individuals. This demonstrated that the size of the foramen magnum in children with achondroplasia were small at all ages, particularly in those with serious neurologic problems (Hecht *et al.*, 1985, Hecht *et al.*, 1989b). Therefore, it was suggested that measurement of the foramen magnum might identify achondroplastic individuals at high risk of developing neurologic complications.

The severely deranged growth plates of the long bones of the upper and lower limbs and in particular the humerus and the femur, result in the disproportionate short stature with significant shortening of the proximal parts of the long bones. The bowing of legs often seen in ACH and in severe HCH, is due to the discrepancy in the growth of the tibia and fibula with the fibula growing more than the tibia (Ponseti *et al.*, 1970).

The final height achieved in males and females with achondroplasia ranges from 118 - 145cms and 112 – 136cms respectively. The mean birth length has been reported as 47.8cms in boys and 47.1cms in girls (Hertel *et al.*, 1994; Horton *et al.*, 1977; Horton *et al.*, 1978) and the mean head circumference in these children is usually greater than two standard deviations above the mean of normal children (Dawson *et al.*, 1980). The frequency of ACH in comparison to other skeletal dysplasias, made it possible to derive disease specific achondroplasia growth charts using auxological measurements from 400 children with ACH (Horton *et al.*,

1978). These growth charts consist of centiles for total height, height velocity and for upper and lower segment measurements. Body proportions are usually described as sitting height (SH) and subischial leg length (SILL), which is calculated by subtracting sitting height from standing height (Tanner *et al.*, 1978). The body proportion measurements are useful in describing skeletal dysplasias with disproportionate short stature of varying severity (Hertel *et al.*, 1994). Disease specific growth charts for the majority of other skeletal dysplasias are currently unavailable and more data on anthropometric data would be useful and help in the evaluation of the natural history of these conditions and to assess response to growth hormone therapy (Okabe *et al.*, 1991; Horton *et al.*, 1992, Hagenas *et al.*, 1996).

Thanatophoric dysplasia (TD) is a lethal disorder and was described as a distinct clinical entity in 1967 and resembles more closely to homozygous ACH with respect to the phenotype, histochemical and radiological features (Maroteaux *et al.*, 1967). TD is at the severe end of the ACH disease spectrum and is divided into types I and II depending on the severity of the cloverleaf skull and the presence or absence of significantly short and curved femurs. Affected children usually die almost immediately after birth due to respiratory failure, resulting from abnormally shaped ribs with a significantly reduced thoracic cavity (Kaufman *et al.*, 1970; Shah *et al.*, 1973).

#### 7.4 Hypochondroplasia: Diagnosis

Hypochondroplasia (HCH, MIM 146000) is inherited in an autosomal dominant fashion with complete penetrance. However in most cases there are no affected family members apart from the index case and presumably this represents spontaneous *de novo* mutations. It is one of the milder variants of chondrodysplasias resembling ACH and the incidence of HCH is unknown. There is a wide spectrum of disease severity, with affected individuals presenting with short stature, mild shortening of proximal ends of the upper and lower extremities and normal facial appearance (Beals 1969; Dorst 1969; Murdoch 1969; Hall 1969 a; Walker *et al.*, 1971; Frydman *et al.*, 1974). As the more severe cases of HCH are mistakenly diagnosed as ACH, it is important to carefully define the clinical and radiological phenotype. This is necessary to prevent further unnecessary investigations and more importantly to reduce parental anxiety, as a diagnosis of ACH has a different prognosis when compared to HCH. The ultimate height potential in HCH ranges from 127cms to 152cms (Beals 1969).

Neurological complications like hydrocephalus and spinal stenosis are well recognised in ACH (Cohen *et al.*, 1967; Hall 1988) but are uncommon in HCH (Beals 1969, Hall *et al.*, 1979)

In 1973, McKusick reported a child born to parents, one with ACH and the other with HCH and described a phenotype that differed from classical ACH and heterozygous HCH. He postulated that ACH and HCH may be allelic disorders. Since then there have been further reports of compound heterozygosity in children born to parents with ACH and HCH (McKusick 1973). Comparison with achondroplasia - clinical presentation, radiology and complications are shown in Table 1.

In both ACH and HCH, an attenuation of the pubertal growth spurt has been demonstrated which has been restored in some cases by the administration of growth hormone (Appan *et al.*, 1990; Bridges *et al.*, 1994; Bridges *et al.*, 1991; Hindmarsh *et al.*, 1991).

#### **7.4.1 Phenotype**

The severe end of the spectrum of HCH is characterised by rhizomelic short stature, relatively normal spine and a short stocky build. They have mild frontal bossing but in the majority of cases the head circumference and facial appearances are normal and this helps in differentiating HCH from ACH

(Hall *et al.* 1979; Beals 1969; Glasgow *et al.*, 1978, Maroteaux *et al.*, 1983).

Macrocephaly as an occasional clinical feature has been reported (Beals 1969; Frydman *et al.*, 1974; Newman *et al.*, 1975). They also lack the mid face hypoplasia seen in ACH. The exaggerated lumbar lordosis is much less

evident in HCH, when compared with ACH. Neurological abnormalities including spinal stenosis and nerve root compression seen in ACH (Horton *et al.*, 1995; Horton *et al.*, 1977; Horton *et al.*, 1978; Oberklaid *et al.*, 1979, Mckusick 1978; Wynne-Davies *et al.*, 1981) have not been reported in HCH. At the severe end of the spectrum of HCH, varying degrees of lumbar lordosis, broad hands and feet are reported (Beals 1969; Glasgow 1978). Mild ligamentous laxity has been described in these patients although motor development is normal (Beals 1969) and as with ACH, cognitive function is preserved in the majority of cases (Glasgow *et al.*, 1978).

A diagnosis of the severe form of HCH is made at an average age of 22 months (Hall *et al.*, 1979; Beals 1969), whereas ACH is a diagnosis that is most often made soon after birth. In most cases of HCH, the disease is sporadic with unaffected parents. However, the clinical and radiological features of HCH in familial and non-familial cases have not been different.

#### **7.4.2 Radiology**

##### ***Skull***

The radiological appearances of the skull are usually normal in HCH. Occasionally they may have mild frontal bossing (Heselson *et al.*, 1979; Specht *et al.*, 1975).

### ***Hands and Feet***

At the severe end of the spectrum of HCH, equal and generalised shortening of the metacarpals and proximal and distal phalanges has been documented. This is in contrast to ACH in whom the second to fourth metacarpals and the proximal phalanges are shortened.

### ***Spine***

Several radiological abnormalities in the spine have been described in HCH. Vertebral changes include posterior scalloping of the lumbar vertebral bodies with shortening of the anteroposterior diameter of the vertebrae but the height of the vertebral bodies is maintained (Heselson *et al* 1979; Specht *et al* 1975).

The interpedicular distance is measured on posteroanterior views from lumbar vertebrae L1 to L5. In normal individuals, the interpedicular distance increases in a cephalocaudal direction from L1 to L5. If the interpedicular distance remained unchanged or decreased from L1 to L5, this is considered abnormal. In HCH, in addition to other radiological abnormalities described, the majority of cases demonstrated this unchanged interpedicular distance (Hall *et al* 1979; Beals 1969; Heselson *et al* 1979). This feature is also seen in heterozygous ACH.

### ***Pelvis***

Shortening of iliac bones, short femoral necks and shallow articulation of the sacrum are documented abnormalities of the pelvis in HCH. This gives the pelvis a squared configuration on x-rays (Heselson *et al.*, 1979).

However, in comparison to ACH, the femoral neck is only slightly shorter and broader and in most of the milder forms of HCH they appear normal (Scott *et al.*, 1976).

### ***Long Bones***

Shortening of the tubular long bones is observed in HCH. The degree of proximal shortening depends on the increasing severity of the disease. In most cases there is a generalised shortening of the long bones, without rhizomelic predominance (Hall *et al.*, 1969a).

**TABLE 1a**

**Comparison Between ACH And HCH**

	Achondroplasia	Hypochondroplasia
Age at presentation	At birth or soon after	variable ( 1 year to puberty)
Inheritance	autosomal dominant . majority sporadic	autosomal dominant . majority sporadic
Incidence	1in 26,000	unknown
Estimated mutation rate	$1.4 \times 10^{-5}$ (Gardner 1977)	unknown
Allelism	Yes	Yes
Predicted height		
males	118-145cms	127- 152 cms (males
females	112-136 cms	and females, Beales 1969).
Phenotype		
Rhizomelic short stature	always	variable
Frontal bossing	present	absent
Mid face hypoplasia	present	usually absent
Genu Varum	usually present	rare (severe group)
Puberty growth spurt	attenuated	attenuated



**TABLE 1b**

**Comparison Between ACH And HCH**

	ACH	HCH
Radiology		
Skull	macrocephaly	normal head size
Odontoid hypoplasia	present	absent
Lumbar spine	narrowing between L1 to L5 ( majority-60%)	parallel between L1 to L5 (invariable)
Vertebral body dimension	decreased	decreased (variable)
Hands	short metacarpals	normal hands
Ileac crest	squared	normal
Neck of femur	splayed metaphysis	short
Long fibula	invariable	2/3rd (severe group)
Thoracolumbar kyphosis	usually present	absent
Lumbar lordosis	invariable	variable
Spinal stenosis	variable severity	absent
Complications		
Neurological symptoms	frequent	Rare
Respiratory problems	common	Absent
Learning disability	uncommon	uncommon

In summary, the following radiological features are considered major criteria in the diagnosis of HCH. These include normal skull with occasional frontal bossing, a generalised shortening of the tubular bones, narrowing or unchanged interpedicular distance between lumbar vertebrae L1 to L5 with anteroposterior shortening of the lumbar pedicles.

A firm diagnosis of HCH can be extremely difficult, especially in the milder cases, despite the clinical and radiological features described above.

Therefore, in addition to having a high degree of suspicion, an experienced radiologist needs to review the x-rays to confirm the diagnosis in the milder cases.

Recently, the disease loci for achondroplasia and hypochondroplasia were mapped to the short arm of chromosome 4. Mutations in the fibroblast growth factor receptor 3 gene has been described in both ACH and HCH (Velinov *et al.*, 1994; Bellus *et al* 1995a., Bellus *et al* 1995b; Bonaventure 1996 a & b).

## 7.5 Fibroblast Growth Factors and FGFR genes

Fibroblast growth factors (FGFs) are a family of multifunctional growth factors with pleiotropic effects on several cell types including mesodermal and neuroectodermal cells (Anderson *et al.*, 1988; Burgess *et al.*, 1989; Mitrani *et al.*, 1990; Thompson *et al.*, 1991). FGFs have been shown to play an important role in embryonal development including cellular growth, differentiation and migration and in angiogenesis (Pasquale *et al.*, 1989; Pasquale 1990; Mitrani *et al.* 1990; Musci *et al.* 1990; Reid *et al.*, 1990; Patstone *et al.*, 1993; Peters *et al.*, 1993). Acidic and basic fibroblast growth factors are implicated in the development of the central nervous system (Anderson *et al.*, 1988; Burgess *et al.*, 1989). The purification of the acidic and basic FGFs was facilitated by their affinity for heparin, which is a hallmark of all FGFs, and there are at least eighteen of these heparin-binding growth factors reported (Burgess *et al.*, 1989). Although acidic and basic FGFs share a significant degree of structural similarities, they are distinguished from each other by their expression patterns. In addition, these growth factors exert their effects on the various developmental processes by binding to different but homologous high affinity cell surface receptors with intracellular tyrosine kinase domains known as fibroblast growth factor receptors (FGFRs) (Dionne 1990; Partanen 1990a; Partanen 1990 b; Keegan 1991a). For example, FGFs 1,2,4,8 and 9 are shown to specifically bind to fibroblast growth factor receptor 3 (FGFR3) (Chellaiah *et al.*, 1994;

Hecht *et al* 1995a; Ornitz *et al* 1996; Santos-Ocampo *et al.*, 1996). They regulate the growth and differentiation of cells through complex signaling pathways.

Fibroblast Growth Factor Receptors (FGFRs) are developmentally regulated transmembrane proteins. The FGFRs constitute a new family of tyrosine kinase receptors (Dionne 1990; Johnson *et al.*, 1990; Hattori *et al.*, 1990). Four fibroblast growth factor receptors (FGFR 1 to 4) have been described (Givol D., 1992). They are membrane bound glycoproteins and share structural homology and have three immunoglobulin like extracellular domains, a transmembrane domain and an intracellular proximal and distal tyrosine kinase domain (Pasquale 1990).

The mouse FGFRs was mapped to murine chromosomes 8, 7, 5 and 13. Comparative gene mapping in the mouse and human fibroblast growth factor receptor genes revealed significant sequence homology. The homologous human loci of fibroblast growth factor receptor genes 1 to 4 are chromosomes 8p11.2 - p12, 10q25.3 - q26, 4p16.3-p15, 5q34-q35 respectively (Avraham *et al.*, 1994).

FGFRs have previously been identified in chicken (Lee *et al.*, 1989) and mouse (Reid *et al.*, 1990). The fibroblast growth factor receptor family genes cloned in chicken include *cek1*, *cek 2* and *cek 3* (Lee *et al.*, 1989; Pasquale 1990) and the respective murine genes described were *flg*, *flg-2*

and *bek* (Reid *et al.*, 1990; Safran *et al.*, 1990; Mansukhani *et al* 1990; Raz *et al.*, 1991; Avivi *et al.*, 1991; Avivi *et al* 1992 a; Avivi *et al* 1992 b)). The human homologue of *flg* and *bek* were FGFR1 and FGFR2. The human homologue of the chicken gene *cek 2* (Pasquale *et al.*, 1989; Pasquale 1990) was identified and named FGFR3 (Keegan *et al.*, 1991a; Keegan *et al.*, 1991b; Keegan *et al.*, 1993). FGFR3 showed 90% homology with *cek 2* gene and 93% homology with the murine homologue *flg-2* (Keegan *et al.*, 1991 a). FGFR3 was shown to bind to both acidic and basic fibroblast growth factors (Keegan *et al.*, 1991 b). FGFR 4, the fourth receptor belonging to the same FGFR family binding to acidic but not basic growth factors has also been described (Partanen *et al.*, 1991).

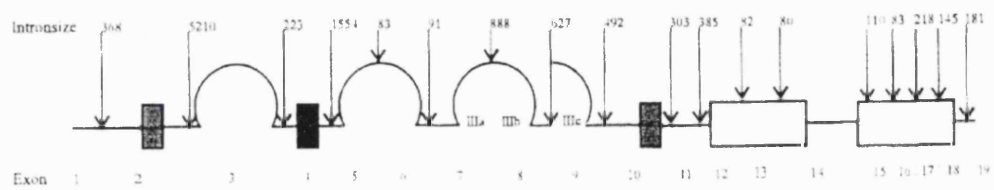
The four FGFRs have a high structural homology consisting of a large extracellular domain containing 3 immunoglobulin-like domains involved in signaling and ligand binding interactions, a single hydrophobic transmembrane domain and an intracytoplasmic tyrosine kinase domain (Partanen *et al* 1991; Givol *et al.*, 1992; Johnson *et al.*, 1993). The 3 immunoglobulin-like repeats in the extracellular ligand-binding domain have been shown to have significant sequence homology to interleukin 1, although the exact functional significance of this domain is unknown (Burgess *et al.*, 1989; Dionne *et al.*, 1990). The transmembrane domain securely anchors the receptor in the plane of the cytoplasmic membrane and hence connects the extracellular ligand-binding domain to the intracytoplasmic region. The juxtamembrane domain separates the

transmembrane domain from the catalytic intracytoplasmic domain and consists of a sequence, which is specific to each subclass.

The kinase domain of subclass IV receptor tyrosine kinase is divided into a proximal half containing the ATP binding site and a distal half containing the catalytic site and is separated by insertions of almost 100 hydrophilic amino acid residues varying in length in different receptors. The inserted sequence has an important role in the modulation of receptor interactions with cellular substrates and the tyrosine kinase domain plays an important role in both signal transduction and in early and delayed cellular responses (Ullrich *et al.*, 1990). Any alteration in the consensus lysine sequence results in inactivation of the tyrosine kinase activity.

## FIGURE 1

### Structure And Organisation Of Human FGFR3



**Figure 1** demonstrates the structure and organisation of FGFR3.

The intronic sizes are shown above the arrows and the exons numbers are shown below.

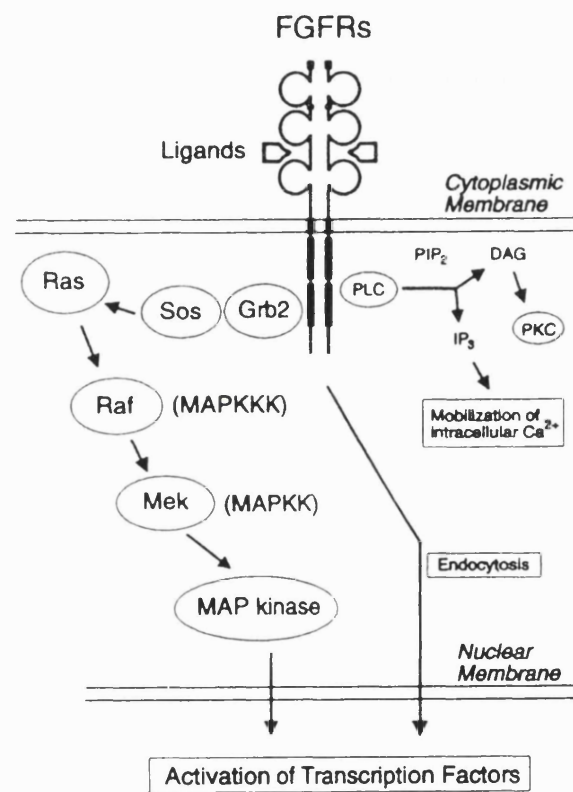
The ligand binding sites of the FGFRs is confined to the extracellular immunoglobulin-like domains two and three (Avivi *et al.*, 1993). The immunoglobulin domain 3 is encoded by three exons, exon IIIa encodes the N-terminal half and exon IIIb or IIIc encodes the C-terminal half. This has been noted in both FGFR1, 2 and 3, whereas FGFR4 was devoid of exon IIIb.

Alternate splicing and variable affinity of specific ligand -receptor pairs results in diverse fibroblast growth factor signaling (Chelliah *et al.*, 1994; Avivi *et al.*, 1993). The activation of the kinase domain following ligand binding and the subsequent conformational alteration of the extracellular domain, induces receptor oligomerisation which is a characteristic feature seen amongst all growth factor receptors. This results in an increase in tyrosine kinase activity and ligand binding affinity. The carboxy-terminal tail (C tail) interacts with the substrate binding sites of the tyrosine kinase domain and therefore alters the capacity to interact with exogenous substrates.



**FIGURE 2**

**FGFRs Signaling Pathway**



**Figure 2** The above figure demonstrates the signaling pathway of the Fibroblast Growth Factor Receptors.

The binding of the growth factors to their receptors is followed by intracellular tyrosine phosphorylation and the receptor aggregates are rapidly internalised.

Following ligand binding and dimerisation, the activated fibroblast growth factor receptor (FGFRs) initiate a sequence of phosphorylation, activating a GTP binding protein Ras and subsequent stimulation of a series of protein kinases and activating transcription factors e.g. Stat 1 in FGFR3. This is shown in Figure 2. The pathway depicted on the right of Figure 2 is a hypothesis that has been suggested as an alternative pathway for receptor signaling. The hypothesis states that the activated receptors could translocate directly into the nucleus and activate transcription factors by endocytosis. However, the role of this process in development is not yet known.

In summary, fibroblast growth factor receptors bind with specific ligands and undergo a complex process of signaling with subsequent activation of transcription factors. In skeletal dysplasias including Achondroplasia, Hypochondroplasia and Thanatophoric Dysplasia, ligand independent activation of the receptor and subsequent transcription regulation results in the varying disease severity.

Mutations in FGFR 1, 2 and 3 have been implicated in human growth, skeletal and developmental disorders (Perez Castro *et al.*, 1997). FGFR4 has not been linked to any disease in humans or animals so far.

## 7.6 Genomic Organisation of murine and human FGFR3

During the search for the Huntington Disease gene on chromosome 4, several novel genes were identified and included FGFR3 gene.

Subsequently, mutations in FGFR3 gene were described in Achondroplasia.

Mutations in FGFR3 gene will be discussed in the next chapter.

The genomic organisation of the murine FGFR3 gene was determined in 1995 following cloning and sequencing (Perez Castro *et al.*, 1995) (EMBL/Gen Bank Data Libraries: Under accession no: L42116 - L42132).

The genomic sequence of the human FGFR3 was described in 1997 (Avraham *et al.*, 1994). Comparative sequence analysis of murine FGFR3 and the human homologue has demonstrated an almost identical structural and genomic organisation.

Both genes (murine and human FGFR3) span approximately 15 to 16.5 KB in length and consist of 19 exons and 18 introns. The boundaries between exons and introns follow the GT/AG rule. It has also been noted that there is a striking similarity in the promoter regions with conservation of several putative transcription factor binding sites between species, suggesting a significant role for these factors in transcription regulation (Perez Castro *et al.*, 1997; Avraham *et al.*, 1994; Wuchner *et al.*, 1997). The translation initiation and termination sites are also very similar and are located in exons 2 and 19 respectively.

One minor and one major transcription initiation codon (ATG) in the brain was identified in exon 2 and one transcription termination codon (TGA) was identified in exon 19. The promoter sequence in both murine and human FGFR3 were within a CpG island and did not have a TATA box. They shared several conserved putative transcription factor binding sites and play an important role in transcription regulation of FGFR3. Several binding sites were located in the 5' flanking region, by using the transcription factor database. Exon-intron boundaries were located by mapping to the cDNA sequence of the murine FGFR3 gene (Avivi *et al.*, 1991; Avivi *et al.*, 1992 a; Avivi *et al.*, 1992 b), which encoded a protein of approximately 800 amino acids.

Alternate splicing and variable affinity of specific ligand -receptor pairs resulted in diverse fibroblast growth factor signaling and has been observed in the immunoglobulin III domain of FGFRs 1 to 3 (Zimmer *et al.*, 1993; Werner *et al.*, 1992; Avivi *et al.*, 1992a, Johnson *et al.*, 1991). The exon/intron structure of murine and human FGFR3 was noted to be identical and the nucleotide sequence homology of the coding region was 85.2% (Perez Castro *et al.*, 1995). This included identical exonic sizes except in exon, 1 and 2 which is known to encode the 5' untranslated region. The sequence homology between murine and human FGFR3 introns ranges between 42% and 58% with the exception of certain intronic sequences including 1,8, 12,13 and 18. These introns form the conserved functional domains that include for example the transcription factor binding sites of

intron 1. The transcription initiation site in the murine FGFR3 was determined by Perez Castro *et al* in 1995, to be upstream in comparison to that described by Keegan *et al* (1991 b) in the human equivalent, which was further downstream of the translation start site. Detailed analysis of the translation initiation site of the human FGFR3 and murine sequence from the 5' untranslated region (Wuchner *et al.*, 1997) demonstrated a 69% sequence homology.

In 1993, cloning and sequencing of FGFR3 between exons IIIa and IIIc identified an alternative exon corresponding to exon IIIb of FGFR 1 and 2 (Chelliah *et al.*, 1994). This additional receptor isoform was shown to have novel ligand binding affinities. It had the hallmarks of the immunoglobulin binding domain and had 44% sequence homology to exon IIIb of FGFR1 and FGFR2 and 36% identity with exon IIIc of FGFR3. This splice variant of FGFR3 (FGFR3 IIIb) binds only acidic FGFs and has the most restricted ligand binding properties when compared with the other fibroblast growth factor receptors. FGFR IIIb has been shown to be expressed in murine skin and epidermal keratinocytes in addition to human colonic epithelium. Expression in colonic epithelium has been demonstrated in both colonic cancer derived cell lines as well as, from normal colonic tissue (Murgue *et al.*, 1994). However, it has been shown that in mice, the overall expression of splice variants IIIc of FGFR3 is greater than exon IIIb. Exon IIIc is expressed in the developing brain, in the spinal cord and very strong expression has been noted in the vertebrae and in all bony structures. Exon

IIIb expression in contrast was only present in epithelial structures with no expression in either the brain or in bony structures (Wuchner *et al.*, 1997).

Stimulation of fibroblast growth factor receptor 3 (FGFR3) results in a variety of functional effects, including regulation of epithelial cell growth and differentiation. In order to characterize the signaling pathway through which FGFR3 regulates cell growth, Kanai *et al* (Kanai *et al.*, 1997 a; Kanai *et al.*, 1997 b) took cells lacking any endogenous FGFR and transfected these cells with the two different human isoforms, FGFR3 IIIb and FGFR3 IIIc, that result from alternative splicing of exon III of the FGFR3 gene, encoding the ligand binding domain. Expression of FGFR3 IIIc in stably transfected L6 cells conferred growth responses to several members of the fibroblast growth family, including fibroblast growth factors 1, 2, 4, and 6, whilst FGFR3 IIIb-expressing cells responded only to FGF1. Activation of FGFR3 upon ligand binding resulted in activation of a protein kinase pathway. Tyrosine phosphorylation of the 66-kDa protein was dependent on ligand activation of FGFR3, suggesting that the 66-kDa protein may play an important role in FGFR3-specific signaling. The activated FGFR3 was found to result in phosphorylation of specific phospholipases.

## 7.7 FGFR Activation and Expression

The action of the fibroblast growth factors is mediated by the four fibroblast growth factor receptors 1 to 4. These receptors have a unique pattern of expression during embryogenesis. Expression of FGFR3 has also been shown to play an important role in various stages of development. This includes expression in developing bone, cochlea, brain, spinal cord (Colvin *et al.*, 1996) and stages of hair growth cycle, particularly during the phase of active hair growth (Rosenquist *et al.*, 1996). Colvin *et al* demonstrated that mice that were homozygous for a targeted disruption of FGFR3 gene developed abnormalities of the bones and inner ear. The skeletal abnormalities included spinal problems including kyphosis, scoliosis and overgrowth of long bones and vertebrae. Inner ear defects included severe abnormalities in the cochlea resulting in profound deafness. These findings, in addition to studying the phenotype of achondroplasia, suggested that activation of FGFR3 would result in the phenotype of achondroplasia (Colvin *et al.*, 1996). FGFR3 expression was also present in the hair cells of the cochlea in the inner ear but was not seen in the sensory epithelial cells of the inner ear.

FGFR3 has a different pattern of expressivity when compared to the other FGFRs i.e. 1,2 and 4. FGFR3 expression in the germinal epithelium of the neural tube occurs between 9.5 to 16.5 days. However, after birth and in the adult brain, expression of FGFR3 was diffuse and localised to

predominantly the glial cells. This is distinct from the expression of FGFR1 in neuronal cells.

Outside the developing brain, FGFR3 expression is predominantly present in the developing long bones. During enchondral ossification, FGFR3 was exclusively expressed in the resting cartilage of the developing bone. In contrast to FGFR1 and 2, FGFR3 expression was not present in other epithelial and mesenchymal tissues. This suggests that FGFR3 is unique in its expression pattern and has a specific role to play during embryogenesis (Peters *et al.*, 1993). This early data suggests that FGFR3 activation is likely to have resulted in the phenotype of achondroplasia and other skeletal dysplasias including hypochondroplasia, that results in rhizomelic short stature. Activated FGFR3 in growth plate cartilage has been shown to result in stunted mice, with hypoplasia of the axial skeleton and craniofacial abnormalities. It was demonstrated that enchondral ossification of bone was inhibited by the activated FGFR3, which caused disruption in growth plate chondrocyte differentiation and proliferation (Naski *et al.*, 1998).

The mouse model of achondroplasia has been developed by disrupting chondrocyte proliferation in growth plates as a result of ligand independent activation of FGFR3. This model confirmed the hypothesis that the phenotype of ACH, is a result of a gain of function of FGFR3 (Wang *et al.*, 1999; Li *et al.*, 1999). Similarly, mutations resulting in thanatophoric dysplasia type II, have been shown to result in ligand independent



activation of the tyrosine kinase receptor of FGFR3. Mutant TDII FGFR3 have been shown to specifically activate transcription factor Stat I, resulting in nuclear translocation of Stat I and this mutant receptor also induces cell cycle inhibitor p21 (Su *et al.*, 1997). The resultant effect is cell growth arrest. This was also demonstrated in tissues of dead TDII fetuses but not in tissues from normal fetuses. The activation of Stat I and induction of cell cycle inhibitor P21 has therefore been postulated as the cause of growth and developmental abnormality in TDII. Similar experiments performed in the mouse model of ACH have confirmed activation of Stat I and p21 cell cycle inhibitors, which resulted in inhibition of chondrocyte proliferation.

Activated FGFR3 targeted to the plasma membrane, results in proliferation of quiescent cells and morphologically transformed fibroblasts (Webster *et al.*, 1997). This suggested that expression of highly activated FGFR3 has the potential to transform cells morphologically and can potentially be tumorigenic. This was further emphasised by the expression of FGFR3 found in 55% of human Kaposi's sarcoma tumour tissues that were examined. Cells transfected with FGF cDNA was injected into thymic mice. Nodular lesions developed at the injection site, which showed a high expression of FGFR3, the histology of which was similar to Kaposi's sarcoma (Li *et al.*, 1998). FGFR3 expression is essential for the normal development of a number of diverse tissues. Li *et al* have also demonstrated the potential tumorigenic role of human FGFR3 although the relevance of this in context to skeletal dysplasia is unclear.

Although there are limited studies with respect to the function of the mutations in FGFR3 resulting in HCH, it has been shown that a similar mechanism described in ACH, results in the severe phenotype of HCH (Webster *et al.*, 1996).

## **7.8 The Genetics of Skeletal Dysplasias**

In the past 6 years, there have been major advances in the understanding of the molecular genetics of skeletal dysplasias (SD), with several loci for chondrodysplasias being identified (Muenke *et al.*, 1995). In 1994, mutations in FGFR3 were identified in ACH (Shiang *et al.*, 1994; Rousseau *et al.*, 1994). Subsequently mutations in FGFR3 have been identified in other skeletal dysplasias included within the achondroplasia disease spectrum. This includes Hypochondroplasia (HCH) and Thanatophoric Dysplasia (TD). The mutations of FGFR3 resulting in ACH and HCH will be discussed separately in the next chapter.

Other gene loci that have recently been shown to have mutations that cause human chondrodysplasias include the collagen related genes, parathyroid hormone related protein receptor genes and those that influence the transport and metabolism of sulfate ions in relevant cells which includes the arylsulfatase E gene and diastrophic dysplasia sulfate transporter gene. The collagen genes encode proteins that occupy cartilage matrix which include collagen molecules II, IX, X and XI and cartilage oligomeric matrix protein

(COMP), that are involved in signal transduction in growth plates (Horton 1997a).

### ***Cartilage Oligo Matrix Protein Gene Mutations (COMP)***

COMP is a member of the thrombospondin family of proteins and is found mostly in the extracellular matrix of cartilage and to a lesser extent in other connective tissues including ligament and tendon. The COMP mutations are predominantly heterozygous and map to regions of the gene encoding the calmodulin repeats. These repeats are important elements of the molecule binding calcium, which is necessary for the correct folding of the molecules (Horton 1997a).

Mutations in the COMP gene were found in Pseudoachondroplasia, a disease that is similar but not identical to ACH (Horton et al., 1995; Horton et al., 1996 a; Horton et al., 1996 b; Francomano et al 1996 b; Horton et al., 1997 a; Horton 1997 b).

At the same time that Pseudachondroplasia was mapped to chromosome 19, linkage to this locus was also established in the Fairbanks Type of Multiple Epiphyseal Dysplasia. Subsequently genomic analysis from these patients showed mutations in the COMP gene and these diseases are considered allelic and presumably share common pathogenic features (Ikegawa *et al.*, 1998). The mutations act either by dominant negative effect or by

haploinsufficiency. It disrupts the normal COMP synthesis and secretion leaving abnormally folded molecules.

### ***Collagen 2A1 Gene Mutations (COL2A1)***

Mutations in the type II collagen genes account for most Spondyloepiphyseal Dysplasia and spondyloepiphyseal dysplasia-like clinical disorders. Mutations in the COL2A1 gene tend to be dispersed throughout the gene unlike mutations in the FGFR3 gene, which are restricted to a few highly mutable codons.

The cartilage collagen fibrils have several types of collagen molecules. Mutations in several collagen genes have shown to result in skeletal dysplasias. Mutant collagen molecules are incorporated into the extracellular matrix of cartilage, resulting in disruption of protein formation. Mutations in COL2A1 gene were first reported in 1989, in a large family with Spondyloepiphyseal Dysplasia (Horton 1997a).

Mutations are dispersed throughout the collagen gene and now there are at least 30 mutations described in the literature. All mutations have been heterozygous involving only one of the two alleles. Subsequently, mutations in this gene were described in Stickler and Kniest Dysplasia (Horton *et al.*, 1997). Mutations in COL2A 1 gene resulting in Spondyloepiphyseal Dysplasia and Stickler Dysplasia, are dispersed across the whole gene as

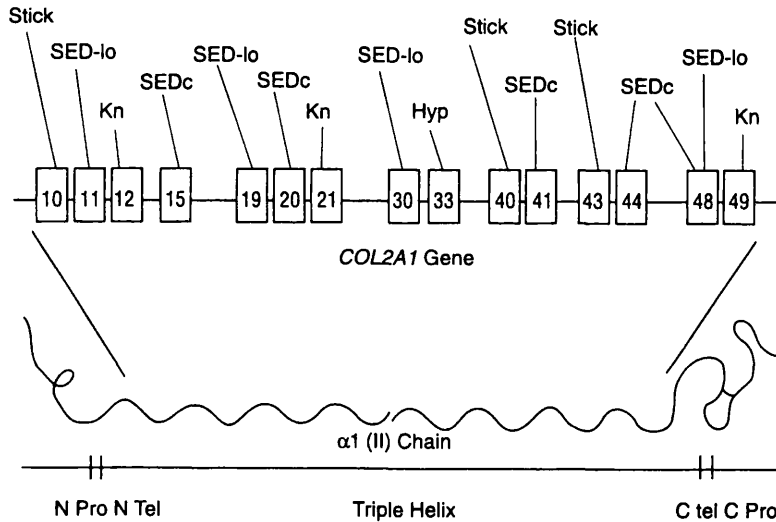
shown in figure 3. Genotype-phenotype correlations are yet to be described in these skeletal dysplasias.

The mutations have a dominant negative effect at the molecular level.

Mutations in Kniest Dysplasia have been shown to interfere with splicing of the mRNA transcripts, resulting in partial or complete deletions of exons that encode parts of the triple helix. This disrupts the assembly of the collagen molecules.

**Figure 3**

**COL2A1 Gene Mutations**



The above is a schematic representation of COL2A1 mutations.

The chondrodysplasias with Spondyloepiphyseal Dysplasia (SED) or SED like disorders with mutations in the COL2A1 gene are depicted above.

**Abbreviations:**

C pro, carboxy propeptide; C tel, carboxy telopeptide; Kn, Kniest dysplasia

Hyp, hypochondrogenesis; N pro, amino propeptide;

N tel, amino telopeptide; SED lo, Spondyloepiphyseal Dysplasia late onset;

SEDc, Spondyloepiphyseal Dysplasia Congenita; Stick, Stickler Dysplasia

The Stickler Dysplasia mutations result in haploinsufficiency with premature translation stop signals. Often the reading frame of the transcript is shifted so that an out-of-frame STOP codon is encountered further downstream. The collagen chains synthesised from such transcripts are truncated and lack the non-collagenous C propeptide, which is necessary for the incorporation of collagen into the triple helical molecules. Early studies had revealed that ACH was not caused by mutations in Type II collagen genes (Francomano *et al.*, 1988)

In summary, COL2A1 mutations are heterozygous mutations and act either through a dominant negative effect or haploinsufficiency. They introduce mutant collagen molecules to the extracellular matrix of cartilage by reducing the amount of protein in cartilage matrix. Since cartilage serves as an important template for enchondral ossification and type 2 collagen is one of the principal structures of proteins of cartilage, it follows that cartilage containing deficient or abnormal type 2 collagen would not function properly as a template.

In several instances suspected allelism has been confirmed in disorders due to COL2A1 and FGFR3 mutations. However, this is surprising in conditions like Pseudoachondroplasia and Multiple Epiphyseal Dysplasia, which are very different phenotypically.

### ***Other genes implicated in skeletal dysplasias***

Mutations in sulphate transporter gene have been implicated in Diastrophic Dysplasia, an autosomal recessive form of chondrodysplasia. The phenotype in this condition is due a loss of function of the sulphate transporter protein.

Mutations in the parathyroid hormone receptor protein has been described in metaphyseal chondrodysplasia, an autosomal dominant condition, with its phenotype resembling vitamin D deficiency rickets. Mutations in the Arylsulphatase E gene and SOX 9, a transcription factor gene structurally related to SRY (sex-determining region Y) gene have been identified in campomelic dysplasias. These are skeletal dysplasias associated with disorders of the external genitalia ranging from minor abnormalities to sex reversal (Horton 1997 a).



### 7.8.1 FGFR3 Mutations In ACH and HCH

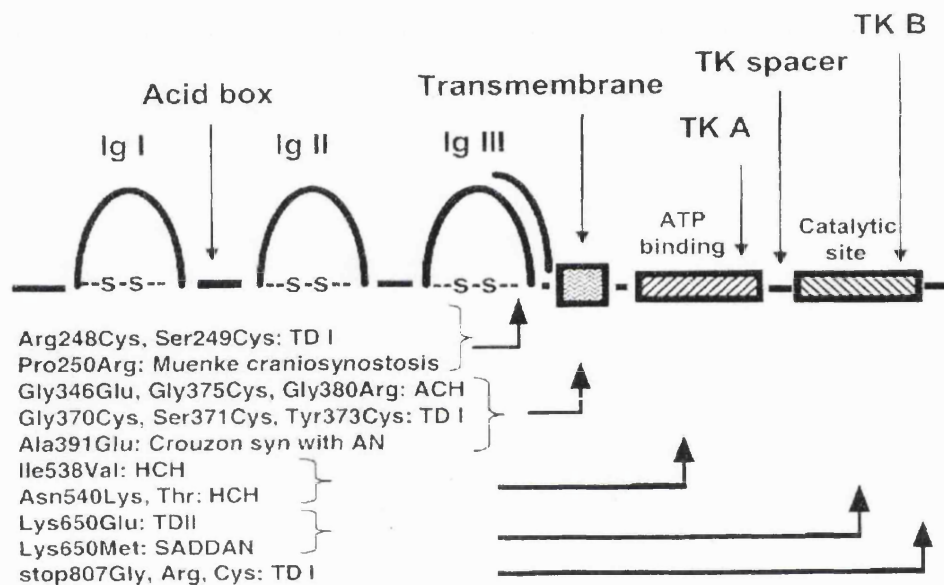
In 1994, the locus for ACH was mapped to a region of about 2.5mb of DNA at the tip of the short arm of chromosome 4 (Velinov *et al.*, 1994). This area had been previously mapped to the locus for Huntington Disease (HD). It was during the search for the HD gene that several novel genes were isolated (Gusella *et al.*, 1983; Thompson *et al.*, 1991; Macdonald *et al.*, 1991; Weber *et al.*, 1991). Nucleotide sequence analysis of one of these genes revealed, that it encoded a member of the fibroblast growth factor subfamily of tyrosine kinase receptors (Thompson *et al.*, 1991; Pasquale *et al.*, 1989; Pasquale 1990). This was subsequently identified as fibroblast growth factor receptor 3 (FGFR3) (Dionne *et al.*, 1990). FGFR3 was shown to be activated by a fibroblast growth factor which was involved in the survival of specific neurons that undergo degeneration in HD (Thompson *et al.*, 1991; Murphy *et al.*, 1990), hence making FGFR3 a candidate gene for HD. However, FGFR3 expression was also seen in several non-neuronal tissues including cartilage rudiments of developing bone, in neurons not affected in HD, including neurons within the substantia niagra, thalamus and cortex. It had also been shown to be exclusively expressed in resting cartilage during enchondral ossification, making it a candidate gene for achondroplasia. This was indeed confirmed by linkage analysis using the  $\alpha$  L iduronidase locus (Francomano *et al.*, 1994; Francomano *et al.*, 1995; Le Merrer *et al.*, 1994; Velinov *et al.*, 1994; Macdonald *et al.*, 1991; Keegan *et al.*, 1993). The other novel genes mapped within the same 3Mb of chromosome 4p,

included the gene for Hurler-Schei Disease (Macdonald *et al.*, 1991) and Congenital Stationary Blindness (Weber *et al.*, 1991, Gal *et al.*, 1994).

Mutations in FGFR3 were subsequently identified in ACH (Bellus *et al.*, 1995 a; Bonaventure *et al.*, 1996 b). Earlier studies had postulated that the IGF-1 gene at chromosome 12 may be a candidate gene for short stature associated with hypochondroplasia (Mullis *et al.*, 1991). This was however proven not to be the case, with mutations in FGFR3 being described in achondroplasia.

**FIGURE 4**

**Schematic Representation Of FGFR3 Mutations**



Schematic representation of human FGFR3 structure with location of common mutations.

**Abbreviations:**

Ig I, II, III, Immunoglobulin Domains I, II and III respectively; TK A, proximal tyrosine kinase domain; TK B, distal tyrosine kinase domain; TDI & TDII, thanatophoric dysplasia II and I; ACH, achondroplasia; HCH, hypochondroplasia; SADDAN, Severe Achondroplasia Developmental Delay Acanthosis Nigricans.

Mutations in FGFR3, resulting in ACH, were first reported in 1994 (Shiang *et al.*, 1994; Rousseau *et al.*, 1994). A single point mutation in FGFR3, resulting in G to A transition at nucleotide 1138 was identified in more than 95 percent of the cases of ACH that they had screened. Two patients had a G to C transversion at the same nucleotide position. Both mutations resulted in a substitution of glycine to arginine at codon 380 (Gly380Arg) in the transmembrane domain of FGFR3 (Bellus *et al.*, 1995 a; Bonaventure *et al.*, 1996 b). Novel mutations in ACH resulting in Gly375Cys and Gly346Glu have been reported in 3 cases of ACH (Prinos *et al.*, 1995; Superti-Furga *et al.*, 1995; Nishimura *et al.*, 1995). Further studies from other countries, including Sweden and Japan, have confirmed the presence of the Gly380Arg mutations in patients with classical ACH. This further confirms genetic homogeneity in achondroplasia, in whom phenotypic homogeneity is also striking (Ikegawa *et al.*, 1995; Alderborn *et al.*, 1996; Wang *et al.*, 1996; Tonoki *et al.*, 1995).

## ***HCH***

Following the description of mutations in FGFR3 in ACH and linkage of HCH to chromosome 4 (Hecht *et al.*, 1995 b), subsequent studies have identified FGFR3 mutations in HCH, which is an allelic disorder with variable disease severity. Heterozygous mutations in FGFR3, with a cytosine to adenine (C to A) or cytosine to guanine (C to G) transition at nucleotide 1620 were identified in a group of HCH patients who were at the severe end of the disease spectrum. These mutations were shown to result in an asparagine to lysine substitution (Asn540Lys) at codon 540 in the proximal tyrosine kinase domain of FGFR3 (Bellus *et al.*, 1995 b; Prinost *et al.*, 1995). There have also been cases of HCH confirmed by radiological diagnosis, in whom, the common mutation in FGFR3 has not been identified (Prinster *et al.*, 1998). Other genetic causes for this group have not yet been described. This further confirms the genetic heterogeneity in this group of patients who are well known for their marked phenotypic variability.

## ***Thanatophoric Dysplasia***

At the severe end of the spectrum of ACH like disorders are thanatophoric dysplasia (TD) types I and II. Mutational analysis of these cases has demonstrated that they segregate genetically into two subtypes. Both subtypes result from mutations in FGFR3. TD II results from a single point mutation in the distal tyrosine kinase domain of FGFR3 and TDI results

from mutations in the extracellular transmembrane domain. At present no case of thanatophoric dysplasia I has been shown to have mutations in the distal tyrosine kinase domain. These mutations in TD, remove the normal translation stop signal. The consequence of this mutation is a FGFR protein that is 141 amino acids longer than normal if translation had continued to the next in frame stop codon (Tavormina et al., 1995).

In summary, despite the complexity and number of skeletal dysplasias described so far, the number of chondrodysplasia loci implicated are relatively few with the majority of mutations in skeletal dysplasias being described in collagen related genes and the fibroblast growth receptor 3 gene. Mutations in Fibroblast Growth Factor Receptor 3 gene cluster within a few codons, ie within the transmembrane and tyrosine kinase domains. This is in contrast to the mutations in the collagen genes, for example mutations resulting in Kniest Dysplasia and Stickler Dysplasia, which are spread throughout COL2A1, with no demonstrable genotype-phenotype correlation. The majority of FGFR3 mutations result in constitutive activation of the receptor in the absence of ligand and studies in mice have shown the disease severity depends on the degree of activation of the receptor (Webster *et al.*, 1996). It is also interesting to note that Wolf-Hirschorn syndrome, a contiguous gene disorder, is caused by different size gene deletions of chromosome 4p16. In addition, although almost always this deletion included the region of FGFR3 to which achondroplasia is mapped, there have not been any cases of Wolf-Hirschorn syndrome with

clinical features of achondroplasia (Shiang *et al.*, 1994; Altherr *et al.*, 1997).

This would suggest that achondroplasia phenotype is probably not due to haploinsufficiency and may be due to other mechanisms, like the presence of dominant negative effects.

More recently, a syndrome associated with severe achondroplasia, developmental delay and acanthosis nigricans (SADDAN) has been described (Francomano *et al.*, 1996 a). A novel mutation in FGFR3 has been identified in some of these cases, which results in an A to T transversion at nucleotide 1949. This resulted in a substitution of lysine to methionine at codon 650. These cases, although have radiological features resembling thanatophoric dysplasia, survive beyond infancy and present with acanthosis nigricans and developmental delay. It is unclear as to the reason why this group of achondroplastic children develop acanthosis nigricans. They are not a group that were treated with growth hormone. Further studies on the expression of FGFR3 in skin and its effect on signal transduction are required.

## 7.8.2 FGFR Mutations In Craniosynostosis Syndromes

FGFR1, FGFR2 and FGFR3 have now been associated with various skeletal dysplasias and craniofacial syndromes. Pfeiffer Syndrome, an autosomal dominant craniofacial syndrome with craniosynostosis, syndactyly and deviation of the thumbs and great toes, results from mutations occurring in both FGFR1 and FGFR2, demonstrating that this is a heterogenous condition (Rutland *et al.*, 1995; Lajeunie *et al.*, 1995; Muenke *et al.*, 1994).

FGFR2 mutations are also described in other craniofacial syndromes associated with craniosynostosis, including Apert Syndrome (Wilkie *et al.*, 1995 b), Crouzon Syndrome (Jabs *et al.*, 1994; Reardon *et al.*, 1994) and Jackson-Weiss Syndrome (Jabs *et al.*, 1994) all of which have distinctive phenotypes although allelic heterogeneity has been described with these syndromes (Jabs *et al.*, 1994; Rutland *et al.*, 1995).

Recently, a new clinical syndrome with coronal craniosynostosis and variable involvement of the hands and feet with developmental delay and sensorineural hearing loss has been described (Bellus *et al.*, 1996 a). This phenotype was associated with a point mutation in the extracellular cellular domain of FGFR3 causing a cytosine to guanine substitution at nucleotide 749. This mutation resulted in a proline to arginine substitution at codon 250 of the FGFR3 protein.



Mutations in FGFR3 have also been described in cases of Crouzon Syndrome associated with acanthosis nigricans. A G to A transition at nucleotide 1172 in the transmembrane domain of FGFR3, resulting in alanine to glutamine substitution at codon 391 of the FGFR3 protein has been described in these patients (Meyers *et al.*, 1995). Interestingly, these patients did not have any radiological abnormalities characteristic of the ACH group of disorders. The mechanism for individual mutations in FGFR3 resulting in specific phenotypes is at present unclear. Further studies to understand the complex signaling pathway of these tyrosine kinase receptors is required to have a better understanding of this group of skeletal disorders.

## 7.9 Growth Hormone Therapy in Skeletal Dysplasias

Growth hormone (GH) is an important regulator of linear skeletal growth, promoting chondrocyte proliferation (Isaksson *et al.*, 1988). Following *in vitro* studies, demonstrating clonal expansion of human chondrocytes in the presence of GH dependent insulin-like growth factor I (IGF-I), it has been postulated that GH increases the responsiveness of the human chondrocyte to IGF-I (Isaksson *et al.*, 1988). It was therefore thought to be a suitable mode of treatment in achondroplasia to promote growth-plate chondrocyte proliferation.

Since the introduction of recombinant human growth hormone (r-hGH) in the late 1980s, its use in other conditions other than isolated growth hormone deficiency has been studied. The background to exploring the wider indications for the use of r-hGH arises from the understanding that growth hormone release does not rely on absolute stature and growth velocity alone (Hindmarsh *et al.*, 1987; Albertsson-Wikland *et al.*, 1988; Spadoni *et al.*, 1988; Saggese *et al.*, 1989). The asymptotic relation between growth velocity and growth hormone secretion suggests that those children who were short and secreted very little growth hormone would have the best response when treated with exogenous growth hormone (Hindmarsh *et al.*, 1987; Hindmarsh *et al.*, 1996).

Growth Hormone therapy has been used in several conditions including skeletal dysplasias like Achondroplasia and Hypochondroplasia (Appan *et al.*, 1990; Bridges *et al.*, 1991; Horton *et al.*, 1992; Bridges *et al.*, 1994; Yamate *et al.*, 1993; Key *et al.*, 1996; Shohat *et al.*, 1996; Weber *et al.*, 1996; Stamoyannou *et al.*, 1997; Tanaka *et al.*, 1998), Turner Syndrome (Rosenfeld *et al.*, 1988; Rosenfeld *et al.*, 1998; Rongen-Westerlaken *et al.*, 1988; Vanderschueren-Lodeweyckx *et al.*, 1990), Osteogenesis Imperfecta (Antoniazzi *et al.*, 1996; Cappa *et al.*, 1991), Downs Syndrome (Anneren *et al.*, 1986; Torrado *et al.*, 1991) and Noonan Syndrome (Ahmed *et al.*, 1991), the one common factor being significant short stature in these conditions.

In the above studies, the use of r-hGH therapy has been shown to be beneficial in these cases although only the short-term response to treatment has been analysed. Previous studies have shown that patients with skeletal dysplasias were not growth hormone deficient (Nishi *et al.*, 1993; Hagenas *et al.*, 1996). Short-term treatment with high dose growth hormone has been shown to improve height velocity (Horton *et al.*, 1983; Nishi *et al.*, 1993). Early reports of r-hGH therapy in ACH showed a sustained, proportionate increase in both spinal and limb growth (Hindmarsh *et al.*, 1991; Bridges *et al.*, 1991). Follow up of these children on growth hormone, has shown accentuation of the existing disproportion, with increase in spinal height in relation to the extremities (Bridges *et al.*, 1994).

The response to growth hormone therapy in the conditions mentioned above have been variable, with some conditions like Osteogenesis Imperfecta

having no improvement in height standard deviation scores in the short term, whilst ACH and HCH demonstrating a favourable response to growth hormone therapy, at least in the short term. Similar responses to growth hormone therapy in Turner Syndrome and Noonan Syndrome have been described. Recombinant human growth hormone (r-hGH) has been shown to promote growth in Turner Syndrome (Ranke *et al.*, 1983; Rosenfeld *et al.*, 1998). The doses of growth hormone used were higher than that used in growth hormone deficiency (12-32 units/m<sup>2</sup>/week).

In the Kabi International Growth Study (KIGS) review of 1993, 923 children with Turner Syndrome were studied. 767 cases of this group were treated with r-hGH alone and the rest were on a combination of r-hGH and estrogen or oxandrolone. The first year response was significant with all treatment modalities. However, at three years of treatment, those on r-hGH alone continued to have significant increases in height velocity. In the other two groups treated in addition with estrogen or oxandrolone, there was a slowing of growth after three years of treatment. In the growth hormone treated group, the total height gain observed was 0.9 to 2.0 standard deviation scores (SDS). This response was more significant in the younger age group (<10 years). This response in the younger children probably reflects the lack of or little catch up growth that is seen amongst the older children on growth hormone.

Noonan Syndrome is an autosomal dominant condition with a phenotype similar to Turner Syndrome. Turner Syndrome and Noonan Syndrome are not growth hormone deficient. Both conditions are thought to have a mild bone dysplasia (Caralis *et al.*, 1974). A significant first year treatment response similar to that observed in Turner Syndrome, was demonstrated in Noonan Syndrome, using higher doses of r-hGH (Cottteril *et al.*, 1993). It was hypothesised that in both conditions there is a partial resistance to growth hormone and therefore higher doses of growth hormone were used to produce better growth rates. There are yet no final height data on these conditions. The KIGS data had analysed a total of 55 patients with Noonan Syndrome. As with Turner Syndrome, the response to treatment with r-hGH therapy over three years was significant and similar improvements in height velocity and height standard deviation scores (HV SDS and HT SDS) were demonstrated. The dose of r-hGH used was also similar to that used in Turner Syndrome and was much higher than that used in growth hormone deficiency (median 28units/ m<sup>2</sup>/week).

The response to r-hGH therapy in HCH was first described in our department (Appan *et al.*, 1990). 20 children with a clinical and radiological diagnosis of HCH were treated with r-hGH therapy. The majority were prepubertal at the start of therapy and remained so until the end of therapy although the treatment period was for only a year. The age range of those treated with r-hGH therapy in their group was 4.3 to 12.8 years. The short-term response to growth hormone therapy in this group was encouraging

with a significant increase in the one-year height velocity from -1.66 standard deviation score (SDS) pre treatment to +1.62 one year after treatment. There was no significant dose-response observed in this study and there were no obvious effects on body proportions in that short duration of treatment period. Subsequently, Bridges *et al* in 1991 demonstrated a significant response in HCH treated with growth hormone. A total of 31 patients were treated with r-hGH therapy over a period of one to three years. The height velocity standard deviation score increments over the first year of treatment were -0.51 to +1.58. In the next two years, although the response to treatment was lower, the HV SDS was maintained above + 0 SDS. However, the majority of these cases were pubertal , presenting with a lack of the normal pubertal growth spurt. Bridges *et al* had demonstrated that giving r-hGH therapy to these children, restored the pubertal growth spurt (Bridges *et al.*, 1991; Bridges *et al.*, 1994).

HCH cases in my study were mostly prepubertal and they will be followed to final adult height and the results of this data will be published when available.

Adverse effects due to treatment with r-hGH therapy have so far been relatively minimal. The common adverse events that have been previously reported include pharyngitis, upper respiratory tract infections, coughing, headache, seizures, fractures, vomiting and rash (KIGS Report 1993). Occasional reports of benign intracranial hypertension have also been

reported (Malazowski *et al.*, 1993). The metabolic problems reported with GH therapy included hypoglycaemia or diabetes mellitus, although both are very rare. There is no definitive data to support the hypothesis that growth hormone treatment increases the risk of malignancy or the recurrence of malignancy e.g. craniopharyngioma, leukaemias or tumours of the central nervous system (KIGS 1993).

## **8. MOLECULAR MATERIALS AND METHODS**

### **8.1 Molecular Reagents**

All chemicals and reagents used in this study were of analytical grade and purchased from standard suppliers, unless otherwise stated.

#### **8.1.1 Stock Solutions**

**100X Denhardts:** Weighed 2g ficoll 400, 2g bovine serum albumin (BSA) and 2g polyvinylpyrrolidene (PVP). BSA was added to 60ml dd H<sub>2</sub>O and left to dissolve overnight without stirring. Ficoll and PVP were added and stirred until dissolved. Made up to 100ml with water. 20 ml aliquots stored at -20°C.

**0.5M EDTA, pH 8.0:** Dissolved 186.1g disodium ethylenediaminetetraacetate.2H<sub>2</sub>O in 800ml of dd H<sub>2</sub>O and stirred vigorously on a magnetic stirrer. Added approximately 20g of NaOH pellets to adjust to pH 8.0. Made up to 1 litre with dd H<sub>2</sub>O, after EDTA had dissolved. The solution was autoclaved prior to use.

**1M magnesium chloride:** Dissolved 20.3g MgCl<sub>2</sub>.6H<sub>2</sub>O in 100ml of dd H<sub>2</sub>O.



**10M sodium hydroxide:** Dissolved 40g NaOH pellet in 60ml of dd H<sub>2</sub>O (in a plastic beaker). Made up to 100ml with dd H<sub>2</sub>O

**20x SSPE:** Weighed 174g NaCl, 31.2g sodium dihydrogen phosphate.2H<sub>2</sub>O and mixed with 40 ml of 0.5M EDTA. Adjusted pH to 7.4 with NaOH (approximately 6.5 ml of 10M NaOH). Made up to 1 litre with dd H<sub>2</sub>O.

**1M Tris (pH7.5):** 12.75g Tris-HCl and 2.36g Tris-Base dissolved in 100ml of dd H<sub>2</sub>O.

**1M Tris (pH 8.0):** 9.76g Tris-HCl and 4.6g Tris-Base dissolved in 100ml of dd H<sub>2</sub>O.

**10X TAE –** Mixed 48.4g Trizma base, 11.4ml glacial acetic acid and 20ml 0.5M EDTA. Made up to 1 litre with dd H<sub>2</sub>O.

**10xTBE - (Tris-borate, EDTA buffer):** Measured 121.1g Trizma base, 51.4g boric acid and 20ml of 0.5M EDTA. Dissolved in 800ml of dd H<sub>2</sub>O on a magnetic stirrer. Made up to 1 litre with dd H<sub>2</sub>O.

**TE Buffer:** 1 ml of Tris pH 8.0 and 0.2ml 0.5M EDTA, pH 8.0 were measured and made up to 100ml with ddH<sub>2</sub>O.

### 8.1.2 DNA Extraction Reagents

**Lysis Buffer:** 0.32M sucrose, 10mM Tris HCl (pH 7.5), 5mM MgCl<sub>2</sub>.6H<sub>2</sub>O, 1% Triton X-100. Weighed 10.94g of sucrose. Added 1 ml of 1M Tris.HCl. (pH 7.5), 0.5ml 1M MgCl<sub>2</sub> and 1ml of 1% Triton X-100. Made up to 100ml with dd H<sub>2</sub>O and stored at 4°C.

**PCR Buffer ( rapid DNA preparation buffer):** with nonionic detergents and proteinase K.

<b>For 100ml</b>	<b>Final Concentration</b>
5ml 1M KCl	50mM KCl
1ml 1M Tris.HCl pH 8.3	10mM Tris
0.25ml 1M MgCl <sub>2</sub>	2.5mM MgCl <sub>2</sub>
1ml 1% gelatin	0.1mg/ml
0.45 ml NP40	0.45% NP-40
0.45ml Tween 20	0.455 Tween 20

Made up to 100ml with dd H<sub>2</sub>O, autoclaved and stored frozen in 20ml aliquots. When ready to use, thawed and added 3µl of 10mg/ml proteinase K (Promega, Southampton, UK, diluted with dd H<sub>2</sub>O ) per 500µl of solution required.

### 8.1.3 PCR Reagents

**10X Polymerase buffer:** 100mM Tris, pH 9.0, 500mM KCl, 1% triton X-100. This solution was supplied by manufacturer (Promega).

**Oligonucleotide primers:** Supplied by Genosys or Oswell. On receipt of primer, 100 $\mu$ l aliquot was taken for working solution. Remainder stored with primer stock solutions.

**Mineral Oil and Sterile Water:** Stored in bijoux bottles. Autoclaved before use.

**Magnesium Chloride:** 25mM stock solution supplied with enzyme.

**DNTPs 100 $\mu$ M concentration (Promega):** Store at -70°C. Prepared stock solution containing 2.5mM of each nucleotide, by mixing equal volumes of the four nucleotides and then diluting the mixture 1 in 40 (final concentration 2.5mM dNTP). Pipetted 1ml aliquots in 1.5ml tubes and stored frozen.

**Taq DNA Polymerase (Promega) :** Supplied at a concentration of 5U/ $\mu$ l in storage buffer [50mM Tris-HCl(pH 8.0), 100mM NaCl, 0.1mM EDTA, 5mM dithiothreitol (DTT), 50% glycerol, 1% Triton X-100]. Stored at -20°C.

#### 8.1.4 Dot Blot reagents

**Denaturing solution:** Weighed 20g NaOH (0.5M) and 87.6g NaCl (1.5mM). Added 800ml of dd H<sub>2</sub>O to above and stirred on a magnetic stirrer. Made up to 1 litre with dd H<sub>2</sub>O.

**Neutralising Solution:** Weighed 87g NaCl and 60.5g Trizma base (0.5M). Made up to 900ml with dd H<sub>2</sub>O. Adjusted pH to 7.4 by adding HCl (approximately 36ml). Made up to 1 litre with dd H<sub>2</sub>O.

**5M TMAC solution:** Dissolved 500g of TMAC in 400ml dd H<sub>2</sub>O. Made up to 912ml with dd H<sub>2</sub>O.

#### **TMAC Hybridisation solution:**

<b>For 100ml</b>	<b>Final concentration</b>
60ml 5M TMAC	3M TMAC
5ml 1M Tris-HCl, pH 7.5	50mM Tris
0.4ml 0.5M EDTA	2mM EDTA
3ml 10% SDS	0.3% SDS
5ml 100X Denhardts	5X Denhardts

Made up to 100ml with dd H<sub>2</sub>O. Just before use, added boiled salmon sperm DNA (Final concentration 100µg/ml).

**Wash solution:** Prepared as hybridisation solution above but without Denhardt's solution and salmon sperm DNA.

**2X SSPE, 0.1% SDS:** Measured 100ml 20X SSPE and 10ml 10% SDS.  
Made up to 1 litre with dd H<sub>2</sub>O.

**Dot Blot Stripping solution:**

<b>For 100ml</b>	<b>Final Concentration</b>
50ml Formamide	50%
25ml 20X SSPE	5X SSPE
1ml 10% SDS	0.1%

Made up to 100ml with distilled water. Heated to 65°C before use.

**Biogel P4 :** (BioRad 150-0450) - for a 10% solution, dissolved 1g Biogel P4 in 10ml 2X SSPE.

### **8.1.5 Electrophoretic reagents and gels**

**10X Bromophenol/Xylene Cyanol buffer:** weighed 0.125g bromophenol blue, 0.125g xylene cyanol and mixed with 30ml dd H<sub>2</sub>O. Gradually added 10g of ficoll 400 (Pharmacia, St.Albans, UK) and then mixed continuously until dissolved. Made up solution to 50ml dd H<sub>2</sub>O and stored in 1 ml aliquots at -20°C.

**Ethidium Bromide:** Dissolved 0.25g ethidium bromide in 25ml dd H<sub>2</sub>O. Mixed on a magnetic stirrer for several hours to ensure that the ethidium bromide had thoroughly dissolved. This was transferred into a universal container and stored in a dark place or wrapped in tin foil. Used at a concentration of 0.5µg/ml, i.e. 2.5µl stock solution in 50ml agarose gel.

**Agarose minigels (2%):** weighed 1g of agarose (Flowgen FMC 50002, Sittingbourne, UK) in a beaker. Added 50ml of 1X TBE and dissolved by stirring on a hot plate. When the agarose solution became clear, added 2.5µl of ethidium bromide, mixed thoroughly and poured into a gel container with two 20 well combs in place. Allowed to set for approximately 30 minutes. The gel was immersed in a gel tank filled with 1X TBE. Electrophoresis was performed at 100mA, 200 V for 10 to 20 minutes depending on the size of the DNA fragment. DNA products were visualised on an ultra violet transilluminator.

**Formamide-dye mix (stop solution):** Mixed 9.5ml deionised formamide (95%), 0.4ml 0.5M EDTA (pH 8.0), 5mg bromophenol blue (0.05%) and 5mg xylene cyanol. Stored in 1.5ml aliquots at -20°C.

**ϕ 174 HAE III DNA marker: (Promega).** Mixed 10µl of marker stock solution, 20 µl 10x Bromophenol blue/ xylene cyanol/ficoll and 70µl distilled water (10% solution). 1-2µL loaded into the wells prior to gel electrophoresis.

**Formamide reagent (SSCP loading dye):**

<b>Solution A</b>	<b>Final Concentration</b>
5ml formamide	95% formamide
100µl 500mM EDTA	10mmol/l EDTA
250µl 1% bromophenol blue	0.05% bromophenol blue
250µl 1% xylene cyanol	0.05% xylene cyanol

**Solution B**

50µl 10% SDS	0.1% SDS
100µl 500mM EDTA	10mmol/l EDTA
4.3ml water	

Mixed A:B :: 3.5ml: 2.5ml.

Aliquoted samples in 1ml tubes and labelled as solution F and stored frozen.

**Pre-cast 48 well 10% polyacrylamide gels:** (Clean gel 48s, Pharmacia Biotech, St.Albans, UK). Precast gels with 10% acrylamide and 2% cross linker were rehydrated for approximately an hour in a buffer (supplied with the gel).

Double and single stranded DNA were detected by silver staining of the gels according to manufacturers instructions (Plus one, Pharmacia Biotech).

**6% Polyacrylamide gels for linkage analysis:** Easigel (Scotlab SL-9213). 25% acrylamide/1,25% bisacrylamide, 20:1, containing 7M urea, 1X TBE - Scot Lab SL-9213, Strathclyde, UK). Stored at 4°C. Easigel diluents (Scotlab SL-9260). Stored at 4°C. 10X TBE - diluted 1 in 10 with dd H<sub>2</sub>O for working solution, 10% dimethyldichlorosilane (Sigma D-3879) in chloroform, TEMED (Sigma T-7024), Ammonium persulphate (Sigma A-9164) - 10% solution made by weighing 0.5g in plastic bijou bottle and adding 5ml dd H<sub>2</sub>O. Stored at 4°C up to one week.

#### **8.1.6 Reagents for autoradiography**

**Universal developer:** Added 50ml of Universal developer PAC419 to 950ml of dd H<sub>2</sub>O and mixed thoroughly. This is usually stable for one week.

**Fixer:** Added 200ml of Ilford Hypam 758249 to 800ml of dd H<sub>2</sub>O and mixed thoroughly. This is usually stable for three weeks.



## 9. MOLECULAR METHODS

### **9.1 Rapid DNA Extraction**

A single sample of 10mls of blood was collected into EDTA bottles from each patient and from their parents when available. DNA was isolated from lymphocytes in all samples, using the "rapid" preparation method (Ehrlich 1989). 0.5 ml of whole blood (5µg DNA/0.5ml blood) was mixed with 0.5ml of red cell lysis buffer in a 1.5ml eppendorf tube and spun at 13,000 rpm for 20 seconds. The supernatant was decanted into 1% sodium hypochlorite solution and the lymphocyte pellet was resuspended in 1ml of the red cell lysis buffer and vortexed vigorously. The above step was repeated two to three times until the solution was free from haemoglobin. The pellet was then resuspended in 0.5ml of PCR buffer with nonionic detergents. To achieve enzymatic degradation of protein, three microlitres of proteinase K (10mg/ml) was added to the resuspended pellet and was incubated at 50-60°C for one hour in a water bath. The solution was then placed on a heating block at 90 degrees centigrade for ten minutes to inactivate the proteinase K. Samples were stored at -20°C in numerical order.

## 9.2 Optimisation of PCR

### 9.2.1 General principles

PCR is an *in vitro* technique, which enzymatically amplifies a specific sequence of DNA. The procedure requires a pair of oligonucleotide primers complimentary to the sense and antisense sequences flanking the DNA of interest (Mullis *et al.*, 1987; Strachan 1996). The single stranded primer sequences of DNA anneal to the template DNA and are extended by a DNA polymerase in the presence of DNA precursors (the four deoxynucleoside triphosphates-dNTPs). The dNTPs are tagged to the 3' hydroxyl end of the primer and form a strand complementary to the template strand. The double stranded DNA so formed is then denatured to make single stranded DNA and the whole process is repeated in a cyclical manner and an exponential increase in the specific DNA sequence has been shown with 30 cycles.

Primer pairs are usually designed to contain an equal number of each of the four bases taking care to avoid repetitive motifs. It is important to avoid complementarity of the 3' (carboxy terminus) ends of the primer pair which would result in a 'primer - dimer' formation. This would result in extension of one primer by the DNA polymerase using either the other primer or itself as the template, resulting in the production of a short and incorrect sequence. The magnesium concentration is important for adequate and specific yield of DNA product, magnesium ions forming a soluble complex with the dNTPs

enabling dNTP incorporation. It also stimulates the polymerase activity and increases the melting temperature ( $T_m$ ) of the double stranded DNA and primer/template interaction.

DNA polymerases are enzymes, which catalyse the synthesis of new DNA strands, which are complementary to the denatured DNA strand of the target DNA sequence. The thermo stable DNA polymerase from *Thermus Aquaticus* is the most commonly used enzyme in the polymerase chain reaction.

### **9.2.2 General precautions**

Primer concentrations were always taken from aliquoted portions and never from stock solutions. Although PCR is a robust technology, care was taken to prevent contamination at all steps and PCR reactions were always performed at specifically allocated benches away from post PCR working areas.

### **9.2.3 DNA amplification**

Primer pairs were designed to amplify exons 12 to 14 encoding the entire tyrosine kinase domain of FGFR3.

PCR work sheet was labelled with the date, primers used, initials of researcher and the relevant sample numbers were documented serially.

Master mix details, thermo cycling and the results were documented in the same sheet. 0.5ml sterile tubes were labeled with the sample numbers and the date.

All reactions were carried out in 25 $\mu$ l volumes.

**TABLE 2**

**Standard Reagents Used In PCR Reaction**

Stock Solution Conc.	Conc. in master mix	Final Conc. per reaction
10X Taq polymerase buffer	2.5X	1X
2.5mM dNTPs	500 $\mu$ M	200 $\mu$ M
25mM MgCl <sub>2</sub>	3.75 $\mu$ M	1.5 $\mu$ M*
Sense primer	2.5 $\mu$ M	variable
Antisense primer	2.5 $\mu$ M	variable
Water	final volume of 10 $\mu$ l master mix per tube.	

\* 1.5mM MgCl<sub>2</sub> is the standard amount used, may be varied.

The standard reagents used in the PCR reaction with the relevant primer pairs in a 25 $\mu$ l volume reaction.

10% dimethylsulfoxide was used in some PCR reactions to improve the quality of the PCR product. To each tube, the reagents were added in the order of 10µl water, 10µl master mix and a drop of mineral oil. DNA (2-5µl of a rapid prep) was added under the oil, to a final volume of 20µl. The samples were spun briefly and then placed on a thermal cycler (Hybaid Ltd, Teddington, UK or Omnigene). They were heated to 98°C for 5 minutes to denature the double stranded DNA. 0.25U of Taq polymerase in 5µl 1x buffer was added to each sample on reaching the annealing temperature. This was then followed by 35 cycles of elongation at 72°C for 30 seconds, denaturation at 94°C for 30 seconds and annealing for 30 seconds (annealing temperatures used: 60°C to 66°C). A final extension step at 72°C for 5 minutes was included.

Optimisation of PCR included magnesium titration, varying thermo cycling, and different primer pairs in mutation negative patients. Magnesium was varied between 0.5mM to 2.0mM MgCl<sub>2</sub> and annealing temperatures of 54°C to 66°C.

**TABLE 3**

**Primer Pairs To Amplify Overlapping TKD Fragments In**

**Mutation Negative Group**

<b>Region amplified</b>	<b>primer pair</b>	<b>product size base pair (bp)</b>
3'end of intron 11 to the end of exon 14	5' ctgagagtgggcgagtttcac 5' AACGTCCACAGCTTCCTCATCAG	573
3' end of intron 11 to middle of intron 12	5' ctgagagtgggcgagtttcac 5' gtcgggacgagacgtgggga	210
end of exon 12 to the middle of intron 13	5' ATGCTGAAAGgtgaggagg 5' gaggagtctgccgacggtcc	266
end of exon 12 to the middle of exon 14.	5' ATGCTGAAAGgtgaggagg 5' AATGGGAACCGGCAT	321
beginning of intron 13 to end of exon 14.	5' tagcggcggtggtgccggt 5'CTGATGAGGAAGCTGTGGACGTT	173

The location of each primer pair on the FGFR3 genomic DNA is shown in appendix 3. Exonic sequence is shown in upper case letters.

## **9.4 Single Strand Conformational Polymorphism**

### **9.4.1 General principles**

Single Strand Conformational Polymorphism (SSCP) is an effective and simple molecular technique that enables detection of point mutations and sequence variations in single stranded DNA. Single stranded DNA will conform in a specific fashion and take up specific sequence- based secondary structures under non-denaturing conditions. Even a single base substitution will alter this conformation. The physical configuration is altered and is detected as a mobility difference on electrophoresis in an acrylamide gel. The sensitivity of this test is altered by the size of the product, the electrophoretic temperature, the characteristics of the gel, the quality of the PCR product etc. (Whittall *et al.*, 1995; Sheffield *et al.*, 1993; Humphries *et al.*, 1997; Sweetman *et al.*, 1992; Hayashi 1998).

### **9.4.2 SSCP Optimisation**

In mutation negative patients, for each set of primer pairs shown in Table 4, SSCP analysis was performed spanning the TKD and electrophoresed on Multiphor II electrophoresis unit.

**TABLE 4**

**SSCP Of Overlapping Fragments Of**

**Tyrosine Kinase Domain Of FGFR3**

<b>primer set</b>	<b>A</b>	<b>B</b>	<b>C</b>
<b>primer pair</b>	SO295/ HchR1	HCH F2/HCH R2	HchF3/SO296
<b>product size</b>	210	266	173
<b>annealing temp(°C)</b>	60	60	62
<b>10% DMSO</b>	added	added	added
<b>DNA</b>	2µl	2µl	2µl
<b>electrophoresis</b>	5°C	15°C	15°C
<b>electrophoresis (min)</b>	10 + 80	10 + 70	10 + 70

1.5 to 2µl of the PCR product was mixed with 5 to 5.5µl of formamide reagent, denatured at 98°C for 3 minutes and snap cooled on ice. Samples were loaded onto rehydrated polyacrylamide gels. Samples were electrophoresed for 60 to 90 minutes depending on the fragment size, i.e. at 200 Volts, 23 miliAmps and 5 Watts for 10 minutes followed by 600 volts,



30 milliamps and 18 watts for 50-80 minutes (or until bromophenol blue reached the anode). When half a gel was used, the voltage was kept the same and the current and power were halved. Gels were then stained with silver. The standard temperature used for electrophoresis was 15°C. If there were no sequence variations or the quality of the results was poor, a second run was undertaken at 4°C . The gels were wrapped in Saran Wrap and visualised on a light box to detect any sequence variations.

## **9.5 Amplification Refractory Mutation System**

### **9.5.1 General principles**

The ARMS method uses three oligonucleotide primers. One primer is complementary to the normal DNA and the other is complimentary to the mutant DNA. The third is a common primer downstream of the two primer sequences. A control primer pair (growth hormone {GH} primer sequences) is also added to each tube. As dot blot analysis involves the use of radioactive methods to detect mutations, it was attempted to perform the ARMS method to detect the C1620A mutation. The primer sequences used are shown in Table 5.

### 9.5.2 Optimisation of ARMS Method

The primer pairs used in this method are shown below in Table 5.

**Table 5**

**ARMS Primer Sequences**

<b>Probe</b>	<b>Stock</b>	<b>Primer sequence</b>
<b>Normal probe (T3729N)</b>	117 $\mu$ M	5' CGGGAAACACAAAAACATCATCAAC <u>C</u>
<b>Mutant probe (T3730M)</b>	63 $\mu$ M	5' CGGGAAACACAAAAACATCATCAA <u>A</u>
<b>Conserved (antisense)</b>	96 $\mu$ M	5' TTGCAGGTGTCGAAGGAGTAGTC

The normal and mutant oligonucleotide probe differs by one nucleotide at the 3' end (underlined). Growth Hormone primers (sense and antisense) were used in every reaction as a control.

**Table 6**

**Arms Master Mixes A and B**

	<b>A</b>	<b>B</b>	<b>final conc. per reaction</b>
10x Taq polymerase buffer	1x	1x	1 $\mu$ l
2.5 mM dNTPs	200 $\mu$ M	200 $\mu$ M	1 $\mu$ l
25 mM MgCl <sub>2</sub>	variable	variable	variable
normal/mutant probe	0.5 $\mu$ M	0.5 $\mu$ M	0.05 $\mu$ l/0.09 $\mu$ l
antisense primer	0.5 $\mu$ M	0.5 $\mu$ M	0.06 $\mu$ l
GH (sense)	0.5 $\mu$ M	0.5 $\mu$ M	0.05 $\mu$ l
GH (antisense)	0.5 $\mu$ M	0.5 $\mu$ M	0.06 $\mu$ l
water	Made up to 5 $\mu$ l master mix volume		

Two master mixes **A** and **B** were prepared as shown in Table 6, using the normal and mutant probes respectively for a final reaction volume of 12.5 $\mu$ l per tube:

Each sample was labeled **A** and **B**. In each tube, the reagents were added in the order of 4 $\mu$ l water, 5 $\mu$ l master mix either A or B and a drop of mineral

oil. 1µl of DNA was then added under the oil to a final volume of 10µl. The samples were spun briefly and then placed in a thermal cycler. Optimisation of ARMS included magnesium titration and varying thermo cycling conditions (Thermocycler: Hybaid Ltd, Teddington, UK or Omnigene). After an initial step of heating at 98°C for 5 minutes, 0.25U of Taq polymerase diluted in 2.5µl of 1X Taq polymerase buffer was added to each sample at the annealing temperature. This was then followed by 35 cycles of elongation at 72°C for (10 or 30 seconds), denaturation at 94°C for 30 seconds and annealing at variable temperature (54°C to 65°C), followed by a final extension step at 72°C for 5 minutes.

## **9.6 DNA Sequencing Using Dideoxy Chain Terminating Method**

### **9.6.1 General principles**

The principle of the Sanger's dideoxy chain terminating method (Sanger *et al.*, 1977) is that a single strand of DNA is synthesised by specific primer sequences and this strand length is terminated when it comes across a chain terminating nucleotide such as ddNTPs (dideoxynucleoside triphosphate). When this reaction is electrophoresed on denaturing acrylamide gels, the pattern of the bands show the distribution of the incorporated dNTPs in the newly formed DNA strand. The principle of the dye priming method is to incorporate a fluorescent labeled dUTP at the 3' end.

### 9.6.2 Sequencing Optimisation

Sequencing was performed using an automated sequencer (Vistra DNA sequencer 725, Amersham, Little Chalfont, UK). The DNA template was purified as described before with gene clean and sequenced using Texas red labeled primer (SO295-TR, Oswell, Southampton, UK) at a concentration of 1 pmol/ $\mu$ l.

The amount of DNA was varied between 1-5 $\mu$ l and the annealing temperature was varied between 50°C to 56°C. For each sample, a master mix was made with 5-10  $\mu$ l of DNA, the labeled primer and water to 26 $\mu$ l . 6 $\mu$ l of master mix was added to 4 tubes each containing one of the four dNTPs (i.e. A,C,G,T). A 25  $\mu$ l PCR reaction was performed at 95°C for 0.3 secs, variable annealing temperature, 72°C for 0.35 secs. A micro titer plate was labelled ACGT and 3 $\mu$ l of loading dye was loaded into each well. To this 8 $\mu$ l of the PCR reaction mixture was added and the mixture was evaporated to a final volume of 3 $\mu$ l and loaded onto the sequencing gel.

The samples were electrophoresed at 26mA, 1.4 kilo volts for 4.5 to 5 hours.

The data was analysed using the software provided with the Vistra sequencer.

## 9.7 Restriction Enzyme Digestion

### 97.1 General principles

Restriction enzyme digestion used in the detection of mutations depends on the ability of each restriction endonuclease to cleave at a specific DNA site, usually 4-6 base pairs in length.

### 9.7.2 Restriction digestion of amplified DNA

A standard reaction contained 5 $\mu$ l of PCR product, 0.5 $\mu$ l (5 units) of restriction enzyme, 1 $\mu$ l of appropriate 10x buffer and 3.5 $\mu$ l of water in a 10 $\mu$ l reaction mix, incubated for a minimum of 4 hours at appropriate temperatures. 5 $\mu$ L of the digested product was electrophoresed on a 2% gel and visualised by ultraviolet transillumination.

The C1620A mutation (Asn540Lys) negative patients were screened for the C1620G mutation (also resulting in Asn540Lys substitution) with *AluI* restriction enzyme digestion as described, and incubated at 37°C overnight.

The sequence variation, found in mutation negative patients on SSCP analysis and subsequent sequencing, was digested with *ApaI* (New England BioLabs 2U/ $\mu$ l) using the method described above.

The C1620A mutation negative patients were also screened for the G1138A (resulting in a glycine to arginine substitution at codon 380 {Gly380Arg} of the transmembrane domain of FGFR3), the common ACH mutation (Shiang *et al.*, 1994). This was done by digesting the PCR product of the transmembrane domain of FGFR3 with restriction endonuclease *SfcI* (New England Biolabs, 2000U/ml).

### **9.8 Microsatellite Linkage Analysis**

Microsatellite markers for FGFR3 D4S227, D4S43, D4S412, D4S1627, FGFR2 D10S190, D10S 221 and FGFR1 D8S278, D8S567 were used to perform linkage analysis in mutation negative patients. The primers were end labelled as follows: 5 µl of oligonucleotide sense primer (10pmol/l), 0.5 µl polynucleotide kinase, 1 µl kinase buffer, 0.5 µl of <sup>32</sup>P-dATP (370MBq/ml, 10mCi/ml, Amersham international, Little Chalfont, UK) and 3 µl of distilled water was mixed by pipetting and incubated at 37°C for 30 minutes. Master mixes for the PCR were prepared as described earlier. 0.1µl of the antisense primer in a reaction volume of 9 µl and 1-2µl of rapid prep DNA were added. Following an initial denaturation step, 1µl of labeled primer/ taq polymerase (per tube: 0.2 µl labeled primer, 0.05µl Taq polymerase, 0.75 µl of ddH<sub>2</sub>O) mix was added to each tube and thermo

cycling was similar to earlier description for genomic DNA. Varying temperatures were used depending on the microsatellites used.

Following thermo cycling, 8  $\mu$ l of stop solution was added to the reaction mixture. Samples were heated at 85°C for 3 minutes.

*6% polyacrylamide gel:* Two glass plates were cleaned with alcohol. The notched plate was sialinised (5% dimethyldichlorosilane in chloroform). The two plates were placed one on top of the other with a spacer (0.4mm) placed in-between the two plates. The gel solution was prepared as follows: 20ml Easigel, 60ml of Easigel diluent, 640 $\mu$ l of ammonium persulphate and 32 $\mu$ l of TEMED were mixed in a beaker and poured carefully in between the plates, care being taken to avoid air bubbles between the plates. A 45 well comb was inserted on the top end of the plate (notched end) and the gel was allowed to set for about 1 hour. The comb was removed and the plates were set up in a sequencing holder (Flowgen, Sittingbourne, UK) filled with TE buffer. The excess urea was removed and the gel was pre run at 50 watts for 15 minutes. 5-10  $\mu$ l of sample loaded was loaded into each well and electrophoresed at 40 watts for 3-4 hours.



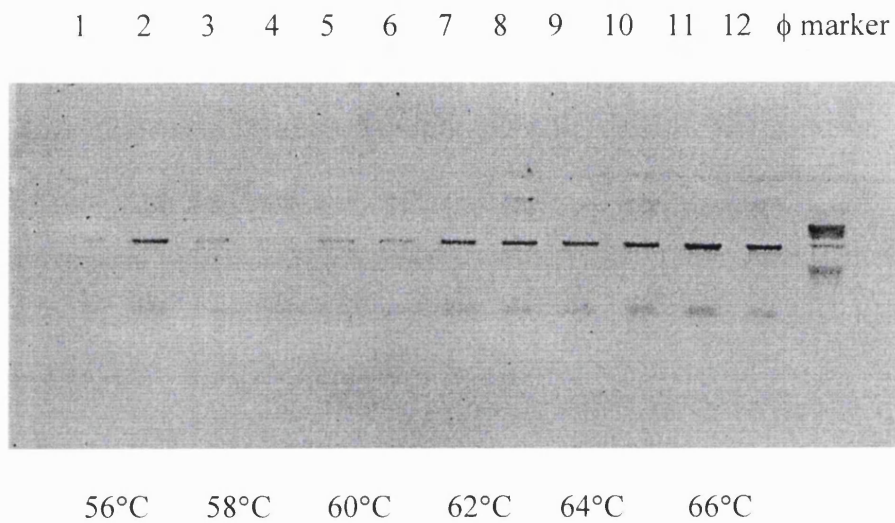
## **10: RESULTS OF MOLECULAR DATA**

### **10.1 Optimisation Of PCR For Tyrosine Kinase Domain Of FGFR3**

Following DNA extraction from 73 patients with HCH and 83 of their parents, the tyrosine kinase domain of the fibroblast growth factor receptor 3 gene was amplified by PCR, using published oligonucleotide primer sequences (Bellus *et al.*, 1995). The amplification of the tyrosine kinase domain proved difficult with persistence of a doublet on amplification of the product. Two PCR products of very similar size were consistently obtained. Increasing the annealing temperature and adding 10% DMSO in the reaction mix resulted in a single band of the correct size.

**FIGURE 5**

**Annealing Temperature Optimisation**

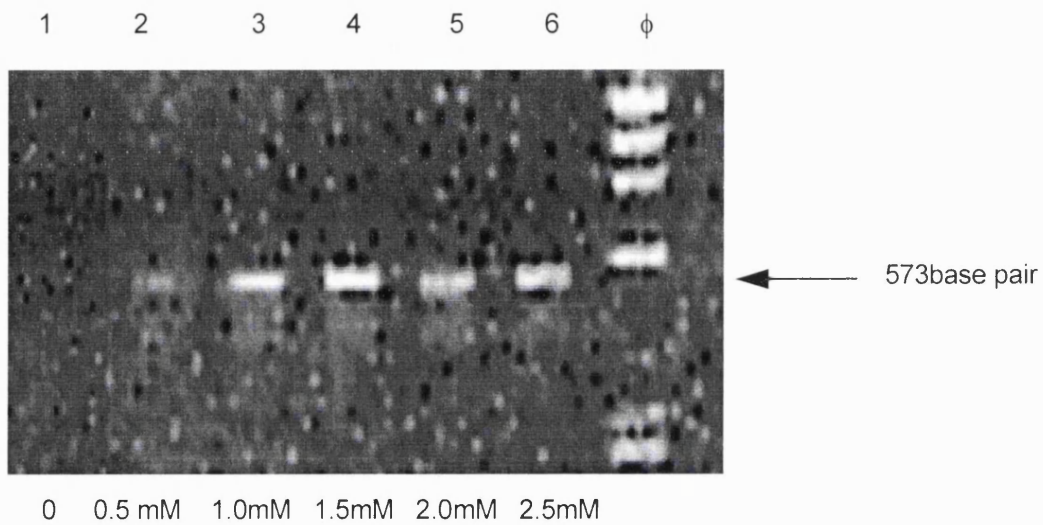


**Figure 5** shows the temperature optimisation of PCR product amplifying entire tyrosine kinase domain of FGFR3. Lanes 1-12: DNA from normal controls used for optimisation experiments. Lane 13 is a DNA marker  $\phi$ . The optimal annealing temperature at which a clean product of the specific DNA sequence was obtained was 66° C.

**FIGURE 6**

**Magnesium Titration of PCR Product**

**(Tyrosine Kinase Domain of FGFR3)**



**Figure 6** Lane 1: no MgCl<sub>2</sub>, lane 2: 0.5mM MgCl<sub>2</sub>, lane 3: 1.0mM MgCl<sub>2</sub>, lane 4: 1.5mM MgCl<sub>2</sub>, lane 5: 2.0mM MgCl<sub>2</sub> and lane 6: 2.5mM MgCl<sub>2</sub>. Lane 7: DNA marker.

Magnesium titration resulted in amplification of PCR product with 0.5, 1.5mM and 2.0mM and 2.5mM of MgCl<sub>2</sub> (figure 6). However 1.5mM MgCl<sub>2</sub> in the presence of 10% DMSO resulted in the best product as shown in lane 4.

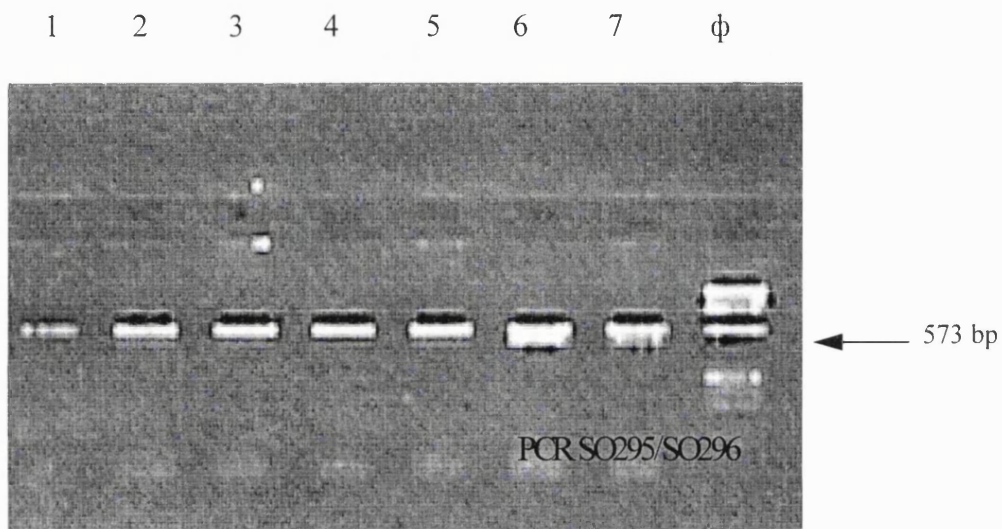
In summary, the optimal amplification conditions for specific amplification of the tyrosine kinase domain were as follows:

25µl final reaction mixture contained 2ml 10X reaction buffer, 2ml of 2.5mM dNTPs, 1.5mM MgCl<sub>2</sub>, 10pmol/µl of sense and antisense oligonucleotide primers. The selected thermo cycling conditions included initial denaturation at 98°C- 5minutes, 66 °C hold at which point the enzyme was added, followed by 72°C- 0.3 seconds, 94°C- 0.3 seconds, 66°C- 0.3 seconds for 35 cycles followed by a final extension at 72°C- 5 minutes.

Results of amplification of 7 DNA samples are shown in Figure 7.

**FIGURE 7**

**Optimised PCR Product**



**Figure 7** Optimised PCR product amplifying TKD of FGFR3 using oligonucleotide primers SO295 and SO296. Lanes 1-7 demonstrate optimised PCR product in 7 HCH patients. Lane 8 is a DNA marker  $\phi$ .

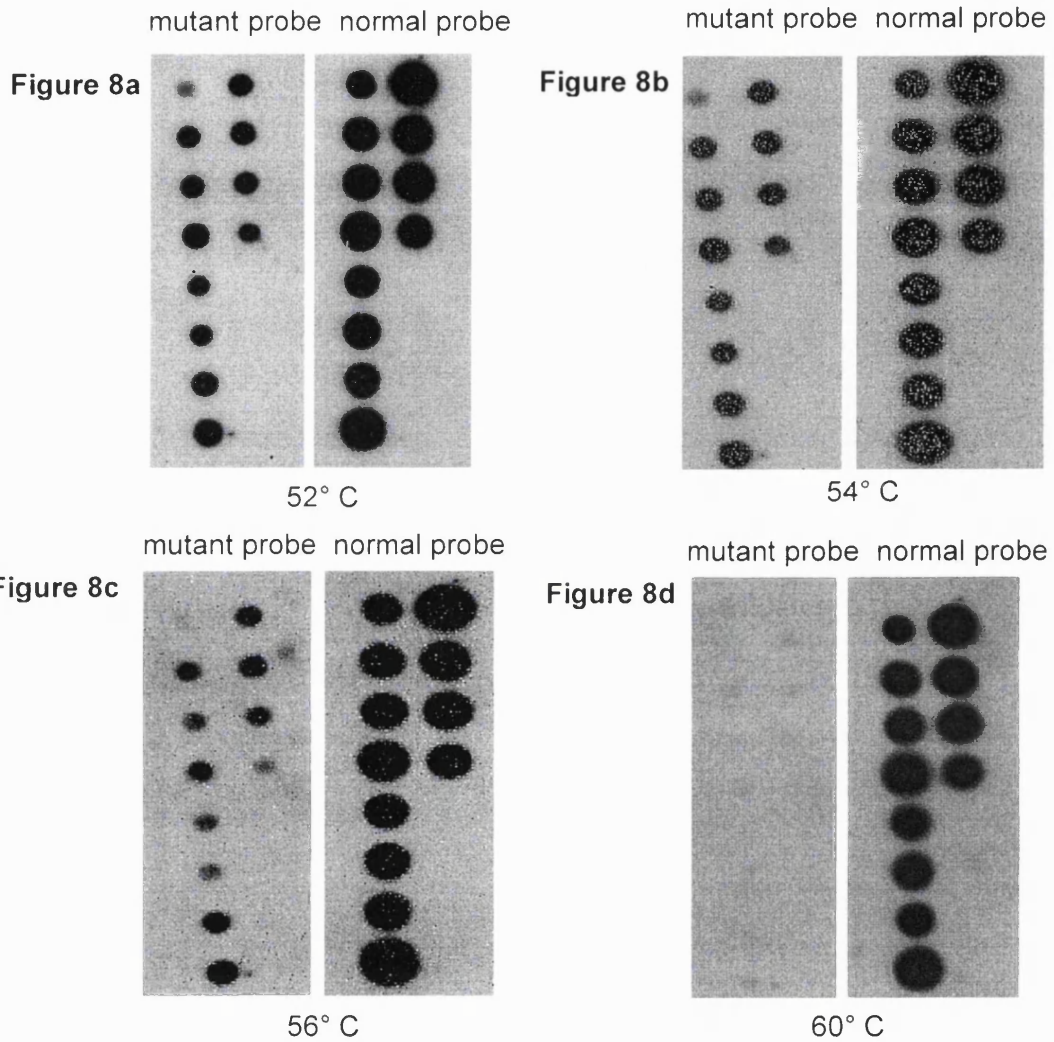
The genomic sequence (Wuchner *et al.*, 1997) was only published after the start of this work and it was not possible to design other primer pairs without including intronic sequences. It was unclear why in the majority of cases, a doublet was present on amplification of the PCR product. The products were very close to each other and were both close to 573 base pairs in length. However, the lower band was much fainter than the upper band. Following hybridisation of the 2 products with an oligonucleotide probe in the TKD of FGFR3, the upper band resulted in a satisfactory signal whilst the lower band produced no signal. The conclusion of this experiment was that the smaller size product was a nonspecific amplification and the assay was optimised to remove this product.

## **10.2 Allele Specific Oligonucleotide Hybridisation (Dot Blots)**

During optimisation, the membranes were initially washed in low stringency washes as described. The temperature of the high stringency wash with TMAC was optimised on normal controls as shown in Figure 8.

## FIGURE 8

### Dot Blot Optimisation



**Figure 8a** High stringency wash with TMAC at 52°C for 15 minutes

**Figure 8b** High stringency wash at 54°C for further 15 minutes.

**Figure 8c** High stringency wash at 56°C for further 15 minutes

**Figure 8d** Optimised dot blot washed at 60°C for 60 minutes

The optimised dot blot analysis for detection of the C1620A (Asn540Lys) mutation was as follows. The amplified PCR product was hybridised with C1620 (Asn 540) and A1620 (Lys 540) P<sup>32</sup> radiolabelled oligonucleotide probes in tetramethyl ammonium chloride (TMAC) as previously described. The hybridised membranes were washed twice in 2XSSPE / 0.1%SDS (0.36M sodium chloride, sodiumdihydrogenphosphate 0.02M, ethylenediaminetetra-acetic acid (EDTA) 0.002M and 0.1% sodium dodecyl sulphate for 10 minutes each (low stringency wash) and subsequently in 3M TMAC at 60°C for 60 minutes (high stringency wash) and autoradiographed overnight.

Dot blot analysis although a robust technique, has some limitations. Care was to be taken when interpreting the signal intensity of the samples. Samples hybridised with the normal and mutant probes were always processed under exactly the same conditions. That is, when hybridising the samples, both mutant and normal probes were subjected to identical hybridisation and washing protocols and autoradiographed at the same time.



### **10.2.1 Dot Blots for Detection of C1620A Mutation**

A normal and mutant control was always included in each assay. As well as being scored by me, an independent observer, my supervisor, also scored the results.

The results were scored as follows.

Homozygous normal: signal with the normal probe only.

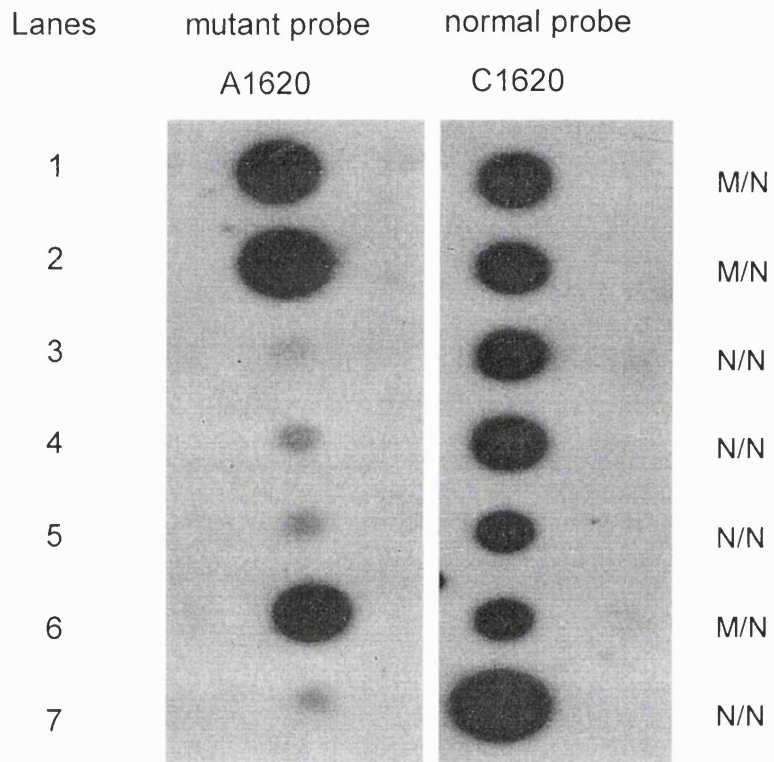
Homozygous mutant: signal with the mutant probe only.

Heterozygous mutant: signal of equal intensity with both the normal and mutant probes.

ASO hybridisation was performed in all 73 patients with HCH. An example of the results obtained on the HCH patients is shown in Figure 9.

**FIGURE 9**

**Detection Of C1620A Mutation Using Dot Blots**



Dot blot analysis demonstrating heterozygous C1620A mutation in lanes 1, 2 and 6.

28 (38%) of the patients with HCH were heterozygous for the C1620A mutation. There were no patients homozygous for the mutation. Of the 83 parents screened, only 5 were positive for the C1620A mutation indicating that the majority of mutation positive cases were spontaneous new mutations. Only one of the five C1620A positive parents was severely disproportionate and had been misdiagnosed as achondroplasia.

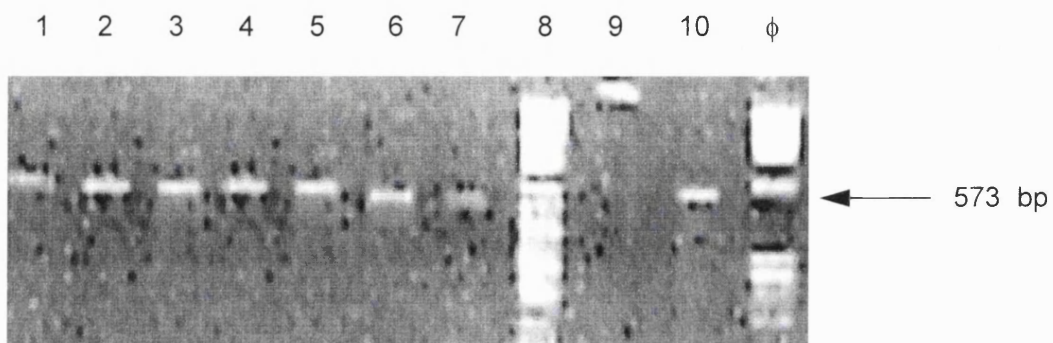
### **10.3 Detection Of C1620G Mutation By Restriction Enzyme Digestion**

The C1620G, which also results in Asn540Lys substitution, creates an *AluI* restriction site in the presence of the mutation (Prinos *et al.*, 1995). The PCR product of the tyrosine kinase was 573 base pairs in length. In the presence of this mutation, the product was cut into a 340 and 233 base pair product, whilst the normal allele remained uncut. To act as a control for the integrity of the *AluI* enzyme, a lambda bacteriophage DNA was also digested with *AluI*. In addition, the uncut lambda DNA marker was loaded as control.

The results of this analysis are shown in Figure 10.

**FIGURE 10**

**Detection of C1620G mutation with *AluI***



**Figure 10** lanes 1 to 7: patient samples, lane 8: digested lambda control; all digested with *AluI*, lane 9: uncut lambda, lane 10: undigested normal control, lane 11: DNA marker.

C1620A mutation negative patients were screened for the C1620G mutation. None of the samples digested with *AluI*, thus indicating that our patient cohort did not have this mutation. The integrity of the enzyme was confirmed with every batch by the cut lambda bacteriophage DNA as shown in lane 8 Figure10.

Digestion of the PCR fragment with *SfcI* in the C1620A mutation negative patients did not reveal the G1138A mutation in the transmembrane domain of FGFR3, the common mutation seen in achondroplasia. Screening for achondroplasia was undertaken to ensure that patients with hypochondroplasia were not wrongly assigned as achondroplasia. The reverse was detected in two patients, who were initially diagnosed as ACH. Both patients were negative for the common mutations described in ACH but further genotype analysis confirmed the presence of the C1620A mutation in both patients and also in the parent of one child who had been misdiagnosed as having ACH.

#### **10.4 SSCP analysis of mutation negative patients**

Further studies of the mutation negative patients included SSCP analysis of the entire tyrosine kinase domain in three overlapping fragments, using oligonucleotide primer pairs that were shown in Table 4. A systematic search for mutations in the tyrosine kinase domain was made in the mutation negative group. The optimal size of PCR product for SSCP analysis is approximately 200-300bp length. Overlapping primers from the genomic sequence were designed to cover the entire tyrosine kinase domain, the product length of each fragment was 210bp, 266 and 173 base pairs respectively (primer sequences SO295/ HCHR1; HCHF2/HCHR2; HCHF3/SO296).

Temperature optimisation of PCR was performed as described previously.

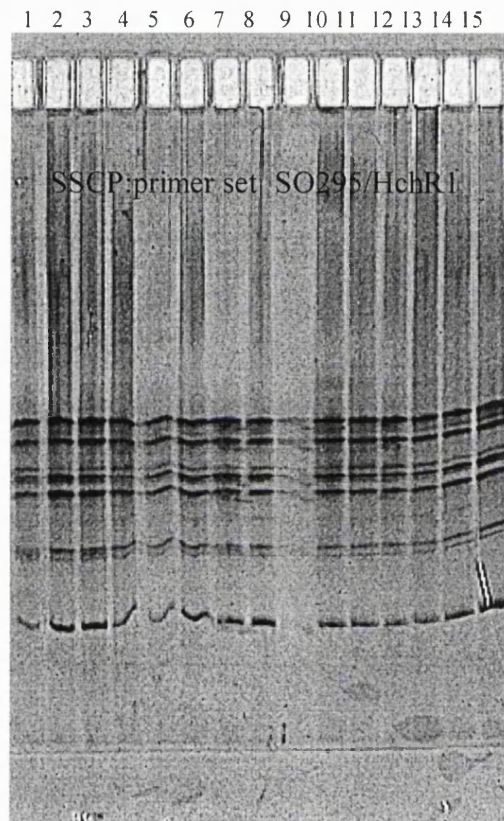
All PCR reactions were carried out with the standard amounts of 10X polymerase buffer, 2.5mM dNTPs, 1 $\mu$ M concentration of sense and antisense primers and 1.5mM MgCl<sub>2</sub>.

Although the standard temperature for electrophoresis was 15°C, a sharper result was obtained at 5°C with primer set A. No definite sequence variation was noted on SSCP analysis using primer sets A (SO295/HCHR1) and C (HCHF3/SO296) shown in figures 11 and 12.

A definite sequence variation was noted on SSCP with primer set B HCHF2/HCHR2 shown in Figure 13. This sequence variation was found in only 4 out of 45 patients (patient id 53, 41, 68 and 50). This was not observed in either 20 normal controls or patients with the C1620A mutation.

**FIGURE 11**

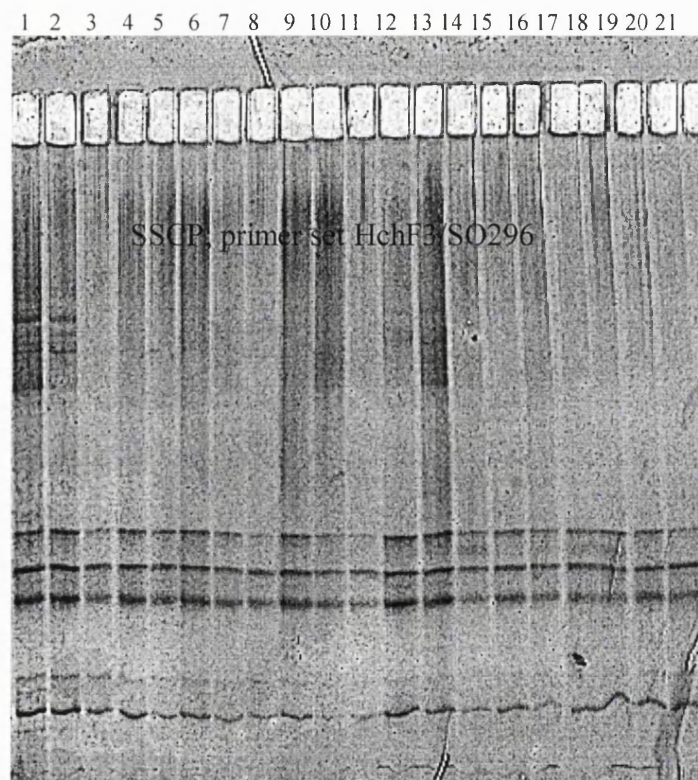
**SSCP Of Tyrosine Kinase Domain Of FGR3 at 5°C**



**Figure 11** SSCP at 5°C of DNA from C1620A negative hypochondroplasia. Lanes 1 to 15: DNA samples from 15/45 HCH patients negative for C1620A mutation, amplified using primer pairs SO295/HCHR1, demonstrating no obvious sequence variation.

**FIGURE 12**

**SSCP Of Tyrosine Kinase Domain Of FGR3 at 15°C**

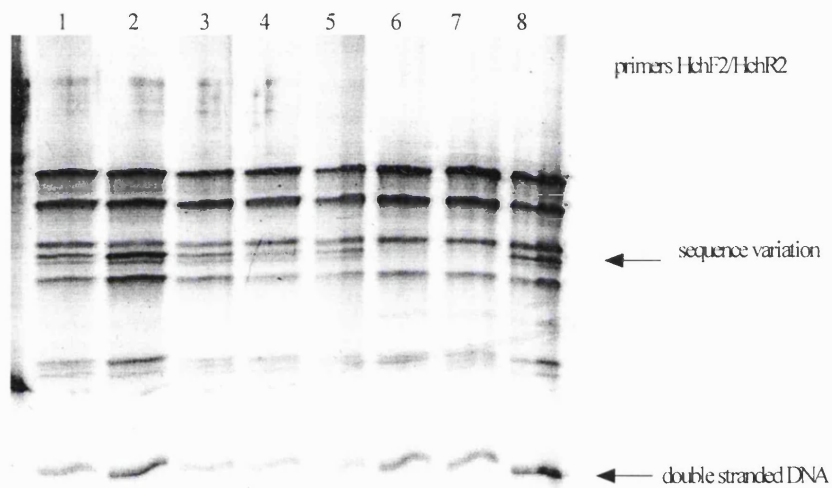


**Figure 12** SSCP at 15°C of DNA from C1620A negative hypochondroplasia. Lanes 1 to 21: DNA samples from 21/45 HCH patients negative for C1620A mutation, amplified using primer pairs HCHF3/SO296, demonstrating no obvious sequence variation.



**FIGURE 13**

**SSCP Sequence Variation**



**Figure 13** SSCP at 15°C of DNA from C1620A negative hypochondroplasia. Lanes 1 to 8: DNA samples from 8/45 HCH patients negative for C1620A mutation, amplified using primer pairs HCHF2/HCHR2. Samples from lanes 6 and 7 demonstrate a sequence variation as shown.

## 10.5 Sequencing of SSCP Variant

Initial problems encountered in Texas-Red sequencing included, excess secondary structure and increased active fluorescence units. Using a template of 1µg and optimizing the primer template ratio to approximately 10:1, resolved this problem. The annealing temperature of 52°C gave the best result and adding 5% DMSO enhanced the quality of the sequencing data. The final PCR reaction used was as follows: denaturation at 95°C, annealing @ 52°C, extension @ 0.35 secs for 25 cycles using Texas red forward primer (SO295) in a 25µl reaction volume. The sequencing of the SSCP variation demonstrated an insertion of a single nucleotide into intron 12 (IIIb form of FGFR3) (Keegan *et al* 1991b) as shown in figure 14.

The normal sequence `gctcctgcacag` is changed to `gctccgtgcacag`.

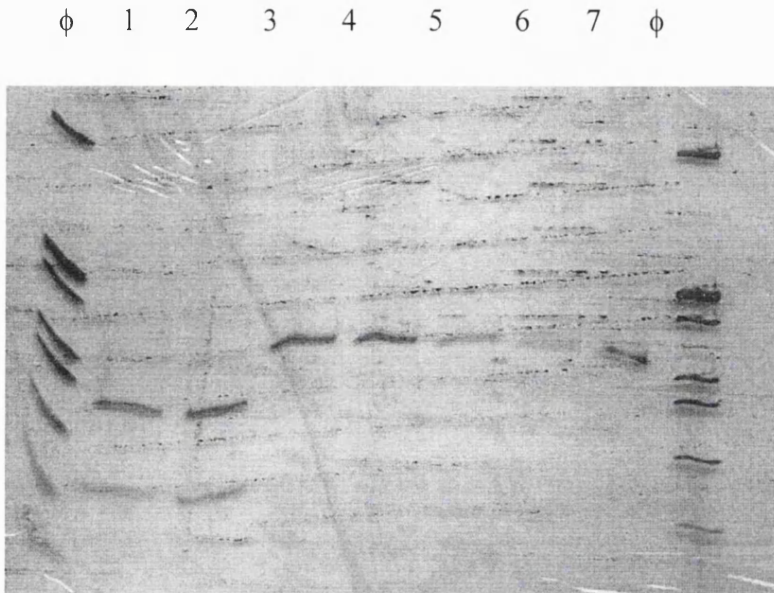
Sequencing was also performed in DNA from two patients in whom the SSCP sequence variation was not found and the above intronic insertion was not detected.



The sequence change removes a cutting site with *Apa1*. That is, the 548 base pair PCR product that would normally cut into 2 fragments of 250 and 290 base pairs in the wild type, is left uncut in the presence of the sequence change. As shown in Figure 15, samples from three patients with the sequence change on SSCP analysis and two controls did not cut. A further 20 normal controls were digested with *ApaLI*, of which 12 were cut as described above in the wild type and 8 were uncut as with the patients with the SSCP sequence variation. It was therefore concluded that the sequence change was a polymorphism of doubtful significance.

**FIGURE 15**

***Apa*LI Digestion of SSCP Sequence Variant**



**Figure 15** Lanes 1 and 8 are DNA markers. Lanes 1 to 4 are normal controls. Lanes 5 to 7 are C1620A mutation negative and had the SSCP variation described previously.

As shown, lanes 3 and 4 (normal controls) and lanes 5 to 7 (mutation negative HCH patients) did not cut when digested with *Apa*LI whilst lanes 1 and 2 (normal controls) cut into two fragments as predicted in the wild type.

The genomic sequence of the IIIc variant of FGFR3 has since been published and it appears that the 4 patients described have the IIIc variant of FGFR3, in which there is an intronic insertion of a G in intron 12 as described earlier (Wuchner *et al.*, 1997). This difference did not appear to have any bearing on the phenotype as these patients were mutation negative patients and were not disproportionate. It therefore appears that there may not necessarily be a difference between the two isoforms, with respect to the clinical phenotype. The underlying molecular cause in these mutation negative patients therefore remains unknown at present.

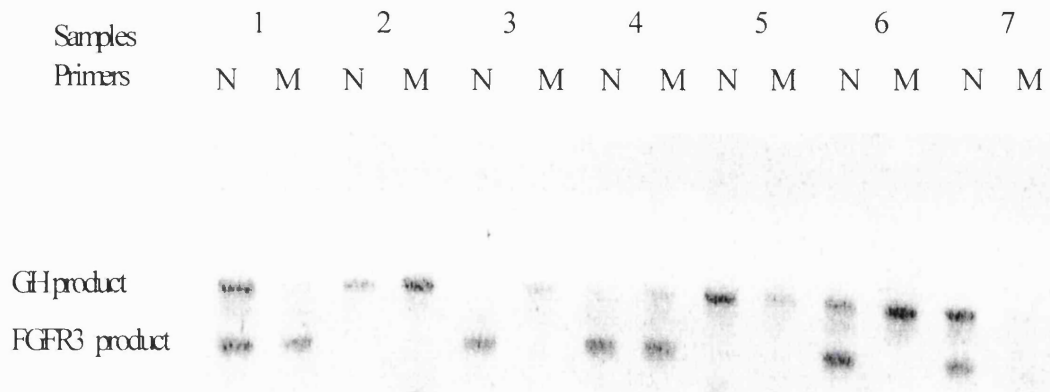
## **10.6 Amplification Refractory Mutation Analysis (ARMS)**

ARMS was tested as an easier method of analysing the C1620A mutation and without the need to use radioactivity. The possibility of forcing a restriction site and performing restriction enzyme digestion was also attempted. However, I was unable to find a suitable restriction enzyme to perform this method.

The size of the G H control product was 450 base pairs and the template DNA product size was 250 base pairs. Although amplification of the sample using the normal and mutant probes and amplification of the GH product was achieved without much difficulty, the ARMS method had numerous difficulties. The amplification was unreliable on several occasions. That is, there were false positive and false negative results. To control for this, a true positive (confirmed by dot blot) and a true negative (normal control) were always included in the experiments. Varied results were obtained when the same sample was amplified on different occasions, despite optimizing and maintaining the same conditions and using the same PCR machine. In the presence of the GH primers, there was an increase in non-specific amplification. However GH on its own resulted in correct amplification of the DNA template. Varied PCR conditions, primer concentrations and template amounts, still resulted in unreliable amplification. An example of the ARMS method after electrophoresing on a 2% gel is shown in figure 16.

**FIGURE 16**

**ARMS To Detect C1620A Mutation**



**Figure 16** For each sample, ARMS products were loaded in the order of normal (N) and mutant (M) primers.

The top lane represents the GH product and should be present in all lanes.

A homozygous normal sample would be expected to give an FGFR3 band with the normal primer (N), likewise a homozygous mutant would see FGFR3 amplification only with the mutant primer (M).

In heterozygotes, amplification occurs with both normal and mutant primers.

Sample 1 and 4 were heterozygous for the C1620A mutation with signals with both normal and mutant primers. Sample 2 and 5 were patient samples and only the GH primer pair amplified. Sample 3 and 6 are homozygous normal, although in sample 3 the GH primer did not amplify. Sample 7 was a normal control. These results were however not reproducible and therefore after nearly 3 months of trying to optimise this method, it was

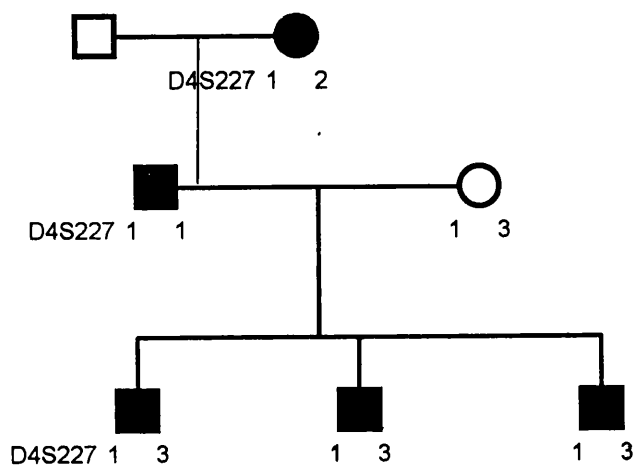


decided to continue the mutation analysis with the established method of dot blots.

### **10.7            Microsatellite Linkage Analysis**

The genomic sequence of FGFR3 was not published during the period of analysis of the C1620A mutation negative patients. In addition, only 4/45 patients had the SSCP variation described previously in section 10.4, which was presumed to be a polymorphism. Therefore it was decided to perform linkage analysis in the mutation negative patients with two or more affected family members. In C1620A mutation negative families, linkage to chromosome 4 was tested by microsatellite analysis. In the majority of cases the disease was sporadic in nature. There were 10 familial cases with either one parent or one or more affected siblings with HCH and linkage analysis was performed in 5 families in whom there was at least three affected family members.

**Figure 17: Example of family with HCH for Linkage Analysis**



**Figure 17** Affected grand mother, father and 3 siblings. The above family was negative for the common mutations of hypochondroplasia and achondroplasia. Linkage analysis for chromosome 4 using microsatellite linkage marker D4S227 was inconclusive.

In one family, 4 half siblings had clinical and radiological features of severe HCH and were C1620A and C1620G mutation negative.

The mother had unfortunately died in a road traffic accident a few years previously; DNA linkage analysis of mum was therefore not possible. Both fathers were clinically unaffected.

The other families were uninformative. The limited number of familial cases and the number of familial cases with more than two affected family members made meaningful linkage analysis extremely difficult.

In summary, 28 (38%) patients with HCH were heterozygous for the C1620A mutation. All patients were negative for the C1620G mutation. In 41 (56%) patients negative for these two mutations, no other sequence variation including the G1138A mutation seen in ACH, was identified by SSCP of the transmembrane and tyrosine kinase domains of FGFR3. In all C1620A mutation negative cases, careful screening of the entire transmembrane and tyrosine kinase domain was performed. A SSCP variant was seen in 4 (6 %) of the mutation negative group and direct sequencing demonstrated a nucleotide insertion in intron 12 which introduced a cutting site with ApaLI. This was also present in some normal individuals, probably reflecting a sequence polymorphism with no functional significance.

**TABLE 7: SUMMARY OF MOLECULAR RESULTS**

**Table 7a**

**Summary of C1620A and C1620G mutation analyses**

	C1620 A positive	C1620A negative	C1620G positive
Patients n=73	28/73	45/73	0/73
Parents n=83	5/83	78/83	0/83

**Table 7a:** The C1620A mutation positive patients all had a severe HCH phenotype.

**Table 7b**

**SSCP in C1620A mutation negative patients**

SSCP analysis n=45	SO295/HCHR1	HCHF3/SO296	HCHF2/HCHR2
No sequence variation	45/45	45/45	41/45
Sequence Variation	0	0	4/45

**Table 7b:** SSCP analysis was performed in the 45 patients negative for the C1620A mutation. SSCP analysis of the entire tyrosine kinase domain was performed, in three overlapping fragments, using oligonucleotide primer pairs as shown in table 4. There were no identifiable sequence variations with primer sets SO295/HCHR1 and HCHF3/SO296. A SSCP variation was noted in 4 patients using primer set HCHF2/HCHR2. This was subsequently sequenced.

## **11. AUXOLOGICAL DATA**

### **11.1 Patient Details**

The children in this study were from endocrine and growth clinics at The Middlesex Hospital and Great Ormond Street Childrens Hospital NHS Trust. The above hospitals are part of the London Centre for Paediatric Endocrinology and Metabolism and are the primary tertiary referral centre for endocrinology in the south east of England. Children under 12 years of age are seen at Great Ormond Street Hospital and those older than 12 years and adolescents are referred to the Middlesex Hospital. Informed consent was always obtained from the parents and from the children where appropriate. Patients presenting with short stature, relative to their family height or disproportionate short stature or any patient suspected to have a skeletal dysplasia had a skeletal survey. All patients who had a definite diagnosis of hypochondroplasia on radiology in the period of January 1982 to March 1998 were included in the study. The radiological diagnosis was confirmed in these patients by two experienced radiologists. A single blood sample was taken for DNA analysis from patients with confirmed HCH and from their parents.

An open study of 35 consecutive children with ACH was undertaken and their response to growth hormone therapy was studied. Comparisons with respect to phenotype and response to growth hormone were made between

ACH and HCH. The diagnosis of ACH and HCH was suspected on clinical grounds and confirmed by standard radiological criteria. DNA analysis was possible in 21 out of 35 cases of ACH in whom blood samples were available. The mutation analysis done previously in this group confirmed the common mutation in all 21 patients.

## **11.2 Auxological Measurements**

Standard auxological measurements were made during each clinic visit. These included height, sitting height and weight. A database was set up (Microsoft Excel, version 5) of all patients with ACH and HCH. At each visit, the chronological age of the patient was calculated accurately as decimal ages (Tanner Whitehouse Growth Charts). Disease specific growth charts for hypochondroplasia are not available. The heights of children with ACH were however, plotted on ACH disease specific growth charts.

At the initial visit, height was obtained from the child and parents. The heights of either or both parents as available were also obtained. The auxological assessments were done by accredited measurers and thus the inter and intra observer variability was kept to the minimum. Height measurements were made using a wall-mounted stadiometer. SH was measured carefully using a wall mounted stadiometer. Subischial leg length was calculated as the difference between sitting height and height. Height velocity was calculated yearly and the value obtained was taken as the

velocity for the midpoint of the year of observation i.e. height velocity of 10cms between ages 9 and 10 was reported as 10cms at 9.5 years. Weight was measured at each visit maintaining standard weighing procedures. Comparisons were made to Tanner normal standards and standard deviation scores were calculated for height, sitting height and height velocity (Tanner *et al.*, 1966 a & b).

SDS was calculated for each measurement using the formula:

$$\text{SDS} = \frac{x - X}{\text{SD}}$$

in which the observed value is x, X is the population mean and SD is the standard deviation of the population at the same age as x.

Similar measurements of height (Ht), sitting height (SH), weight (Wt), subischial leg length (SILL) and height velocity calculations (HV) were made before treatment and 6 monthly thereafter in our cohort of patients with achondroplasia. These measures were converted to age and sex appropriate SDS by comparison with normal reference standards (Tanner, 1966 a & b).

Comparisons were made with achondroplasia patients with the G1138A mutation. Group 1 - ACH with G1138 A mutation, Group 2 - HCH with C1620A mutation and Group 3 C1620A mutation negative HCH. The



comparisons were made to assess differences in responses to r-hGH therapy between ACH and the heterogenous group of HCH, presenting with varying degrees of disease severity.

### **11.3 Disproportion Score (DPS)**

During my clinical examination and data collection of HCH patients, it became apparent that there was a phenotypic spectrum with respect to the disproportion, i.e it became clear that in fact some children who had the radiological criteria of HCH, had shorter backs than legs, instead of the classical rhizomelic limb disproportion. By analysing the raw data and comparing SH SDS and SILL SDS, I was able to divide HCH patients into 3 groups depending on their disproportion. A disproportion score was derived by subtracting SILL SDS from SH SDS.

A score of 1 ( $\text{SHSDS} - \text{SILLSDS} \geq +1$ ) was obtained in those who had the classical presentation at the severe end of the disease spectrum, with rhizomelic shortening, i.e. short limbs and normal or relatively spared spinal length.

A score of 3 ( $\text{SHSDS} - \text{SILLSDS} \leq -1$ ) was obtained in those cases, in whom the spinal length was significantly shorter than the limb length.

Score 2 ( $\text{SHSDS} - \text{SILLSDS} \leq +1$  to  $\geq -1$ ) was obtained in whom there was relatively little disproportion and there was more or less an equal shortening of the back and limbs.

#### **11.4 Pubertal Assessment**

Puberty was assessed according to Tanner (Tanner 1962; Tanner 1976), at each visit. The puberty rating was expressed as a score, which included development of external genitalia in boys and breast development in girls, pubic and axillary hair development. In boys testicular volume was measured using Prader's orchidometer. The onset of puberty was rated as 4ml testicular volume in boys and breast stage 2 in girls. For analysis the patients were divided into 4 groups according to the stage of puberty. Stage 1 prepubertal, stage 2 testicular volume 4 to 6mls or breast stage 2, stage 3 testicular volume 8-12mls or breast stage 3-4, stage 4 testicular volume greater than 15 mls or onset of menarche.

#### **11.5 Bone Age Assessment**

In this study, bone age and height predictions were not performed routinely because the abnormal bones in ACH and HCH prevent interpretation of x-rays for bone ages. A similar problem is also seen in other skeletal dysplasias. Unlike patients with short stature due to growth hormone deficiency or familial or idiopathic short stature, patients with skeletal dysplasia have abnormal bone architecture, which makes bone age assessment difficult and unreliable (Cox 1996). This is particularly so at the more severe end of the spectrum of the disease. The inter and intra observer

error even in the normal situation, is well documented (Breunen *et al.*, 1980).

As our patient cohort were heterogenous with respect to disease severity and bone age assessments are unreliable as discussed, comparisons were made with historical controls to assess the response to therapy. This has its limitation and with the secular trend is not necessarily the ideal way to assess responses to treatment. Such a method has been used to assess the treatment effects in other conditions and carries with it the potential of misrepresenting final outcomes if there has been a significant secular trend, as has been demonstrated in the normal population (Hindmarsh *et al.*, 1987; Hindmarsh *et al.*, 1996) and in Turner Syndrome (Taback *et al.*, 1996; Hertel *et al.*, 1994; Freeman *et al.*, 1995; Lyon *et al.*, 1985). Historical comparison of data was possible with ACH in whom the phenotype was homogenous with little variability. Our data suggest that our subjects were comparable to historical controls at the time of treatment and that comparisons are valid. The magnitude of the secular trend in normal subjects is approximately 2 cms and is unlikely to be a significant factor in the growth outcome of these children (Freeman *et al.*, 1995).

### **11.6 Growth Hormone Therapy**

HCH patients growing slowly with HV SDS less than the -0.8 SDS (below the 25th velocity centile) were commenced on recombinant human growth hormone (r-hGH). The majority of patients in this study were prepubertal.

This was therefore a different group of patients from previous studies, where the majority of patients presented at the time of puberty with pubertal growth arrest (Appan *et al.*, 1990). Growth Hormone was administered as daily subcutaneous injections at night. Dynamic growth hormone testing was not performed in these patients. Studies in the past, have clearly demonstrated that patients with skeletal dysplasias are in fact, not growth hormone insufficient (Bridges *et al.*, 1994; Bridges *et al.*, 1991). The range of growth hormone doses used was 16 to 44U/m<sup>2</sup> / week with a median dose of 30U/ m<sup>2</sup> / week.

A similar dose of r-hGH therapy was used in our cohort of patients with ACH. One needs to mention that the dose of GH used in this study is effectively that of a long term study of patients treated initially with either 20 or 40U/m<sup>2</sup>/week for the first two years and some were arbitrarily randomised to 30U/m<sup>2</sup>/week and this largely reflects the personal practice within the department. However there was little difference in pretreatment Ht SDS within groups treated with either low or high dose r-hGH therapy in the HCH and ACH cohorts.

### **11.7 Statistical Methods**

Ht, SH, SILL and HV data were expressed as SDS with respect to Tanner normal values (Tanner *et al.*, 1966 a,b). SPSS v7.0 statistical package was used for analysis. Examination of data was undertaken to confirm normal

distribution. Differences in group mean data were compared by one way analysis of variance (ANOVA) and Student Newman Keul or Scheffe's post hoc tests for multiple contrasts. . The post hoc test is set to avoid a type I statistical error (false positive result) to less than 5%.

This method was used to compare two or more data sets in either a single sample or between samples. Two way ANOVA was used to compare multiple data points of the same individual. To compare the variances, the ratio of the above two parameters is expressed as F.

Stepwise multiple regression was used to ascertain the effects of age at therapy and dose of growth hormone on the auxological response, this being expressed as a changes in both Ht and HV SDS. Lowess Locally Weighted Regression scatter plot smoothing procedure was used to demonstrate the HV SDS curves in treated and untreated patients with ACH (Cleveland *et al.*, 1979). This uses an iterative weighted least squares method to fit a line to a specified percentage of points and the default of 50% was used in this study. Data are expressed as median and range in the text and shown as mean and SEM in the figures.

## **12. AUXOLOGICAL DATA RESULTS**

### **12.1 Patient Details**

73 patients presenting to the growth clinics fulfilled the radiological criteria of hypochondroplasia with decreased interpedicular distance between lumbar vertebrae L1-L5 with short pedicles. There were 44 males and 29 females. The mean age at presentation was 9.4 years. The age of presentation in the mutation positive and negative groups were 5.8 and 10.45 years respectively. The ACH patients were much younger with a mean age of 3.6 years at presentation. The majority of patients in this study were prepubertal at the time of data collection and start of growth hormone therapy. In HCH, 49 patients were prepubertal, 12 were in early puberty, 8 patients were in mid puberty and 4 patients had a testicular volume of 15mls or had attained menarche.

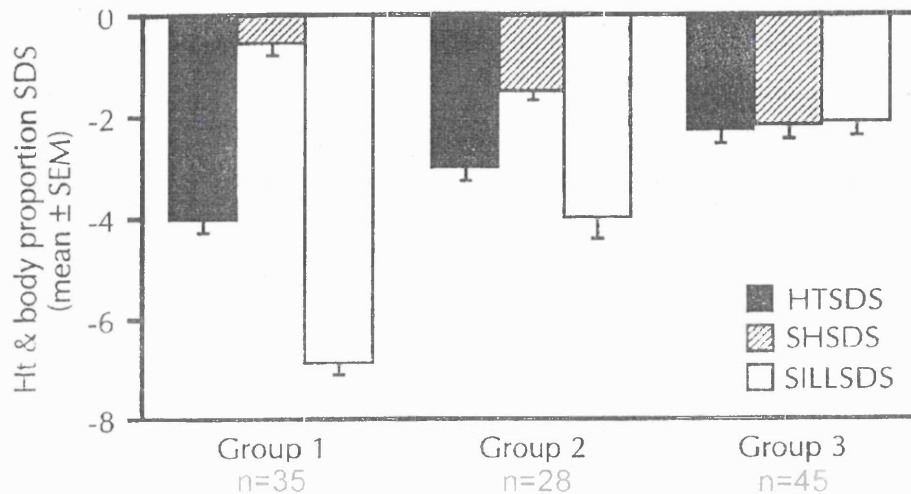
### **12.2 Auxological Measurements**

Patients were divided into 3 groups for comparison of auxological measurements: Group 1 ACH with the G1138A mutation, Group 2 HCH with C1620A mutation and Group 3 HCH with no mutations identified. There was no significant delay in the onset of puberty compared to the normal population in the older children in either group.

The pretreatment baseline growth data of the 3 groups are shown in figure 18. The children with ACH were much shorter than those with HCH, but all patients were significantly compromised in height with respect to family heights ( $p < 0.05$ ) as shown in figure 18. ( $p < 0.05$ ). In ACH, this finding was almost solely due to shortening of the legs. In comparison however, there was a much more variable phenotype in HCH with patients presenting with varying degree of disproportion. Significant differences in SH SDS and SILL SDS were noted between the three groups ( $p < 0.001$ ). The mutation positive HCH group was very similar to the ACH group, although they were relatively less severe and had additional shortening of the back. Group 3 was associated with proportionate shortening with an almost equal reduction in both SH and SILL as shown in figure 18.

**FIGURE 18**

**Baseline Auxology In Achondroplasia And Hypochondroplasia**



**Figure 18** Group 1 ACH; Group 2 HCH with C1620A mutation;  
Group 3 C1620A mutation negative HCH patients.

Ht SDS, height standard deviation score; SH SDS, sitting height standard deviation score; SILL SDS subischial leg length standard deviation score  
Group 1 (ACH) and 2 (HCH) had similar auxological presentations.  
However, in HCH with the common mutation, the severity of rhizomelic short stature was less.



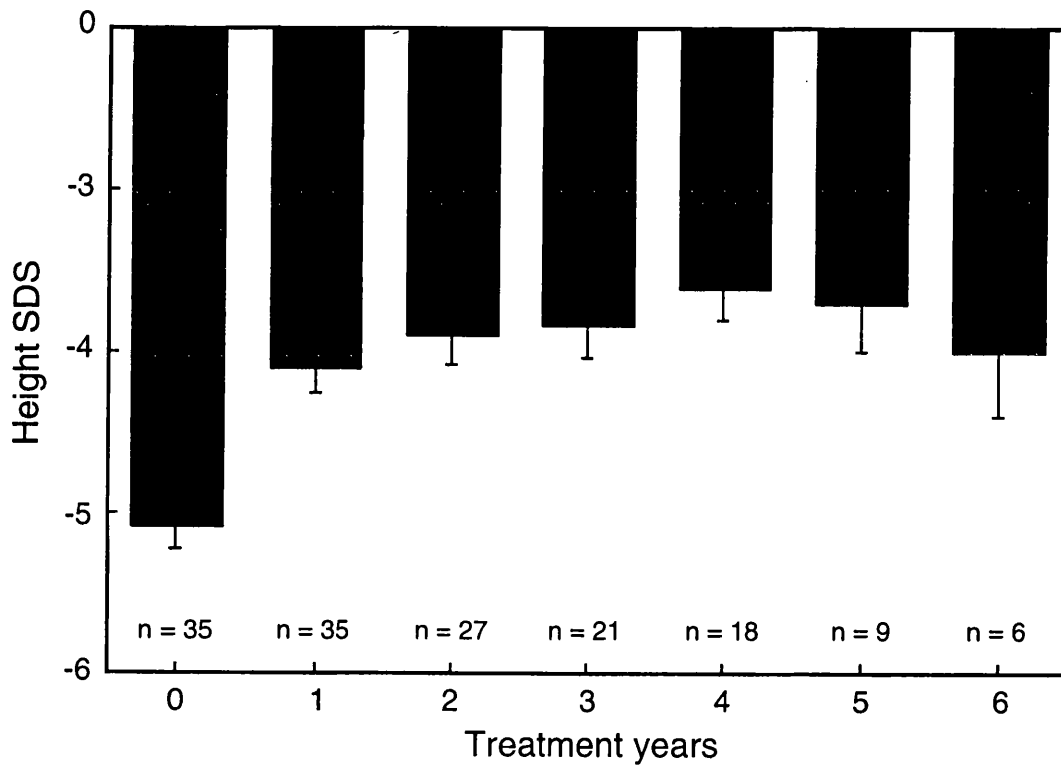
### **12.3 Achondroplasia and Treatment With r-hGH**

35 children with a confirmed diagnosis of achondroplasia were treated with r-hGH for a period of 1 to 6 years. The short stature in ACH was almost solely due to a reduction in leg length and is reflected in the decreased SILL SDS. Analysis of auxological data was easier because there was phenotypic and genetic homogeneity in analysis of this cohort. The median age of the group was 2.25 (1.2 to 9.3 years). They were prepubertal at the start of therapy and most patients remained prepubertal during the course of assessment of response.

Over the six year period the increase in Ht SDS was significant and this increase was predominantly due to an increase in SH SDS with minimal increase in SILL SDS (Figures 19 & 20).

**FIGURE 19**

**Ht SDS In Achondroplasia Over 5 Years Treatment With r-hGH**

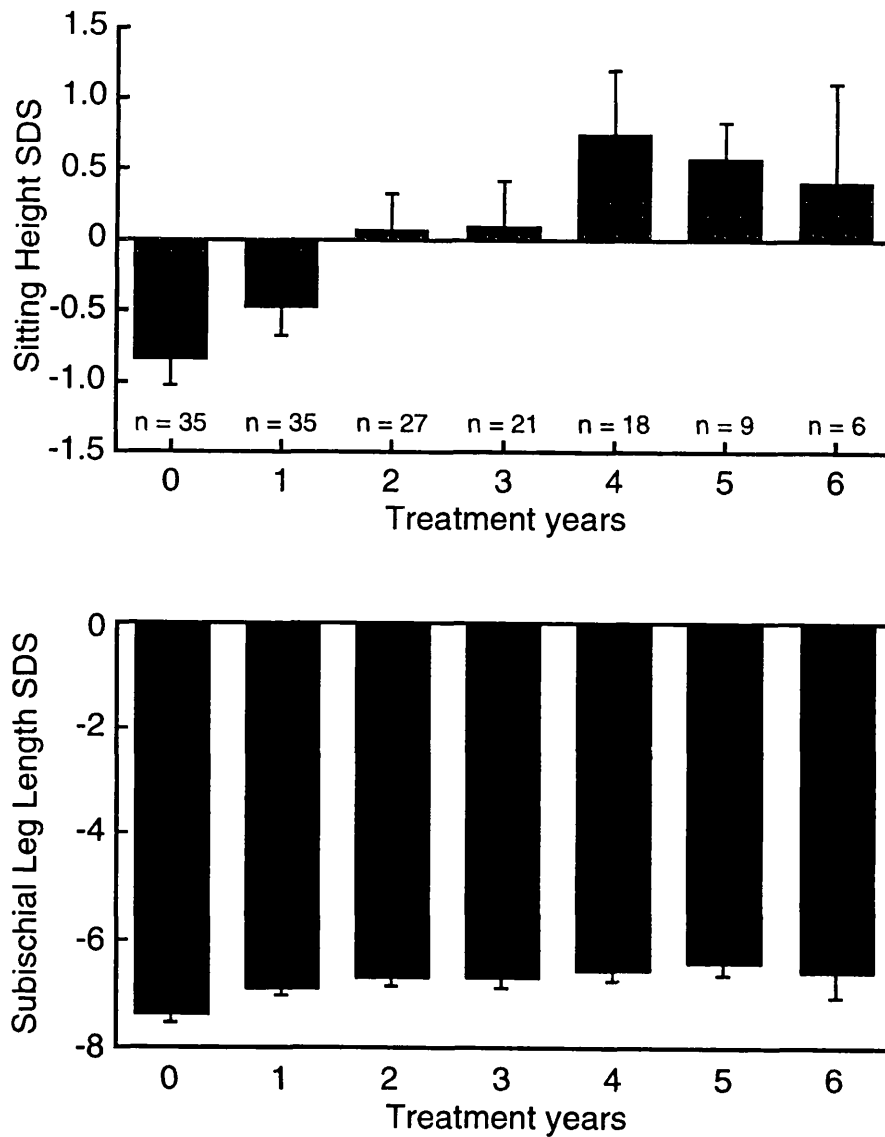


**Figure 19** Change in Ht SDS over time, during r-hGH therapy. Serial Ht SDS in 35 children during 6 years of treatment with r-hGH.

A progressive increase in Ht SDS from baseline to year 4 was noted (F 46.94;  $p < 0.001$ ). Year five and year although different from baseline, were not significantly different from year 4 ( $p > 0.05$ ) but the numbers are small by then. The numbers simply reflect the increasing frequency of recruitment over the past few years.

**FIGURE 20**

**Body Proportions In ACH On Treatment With r-hGH**



**Figure 20** Effect on body proportions. Stepwise year on year increase in SH SDS (upper panel;  $F_{26.25}$ ,  $p < 0.01$ ) and SILL SDS (lower panel;  $F_{9.04}$ ,  $p < 0.01$ ) from baseline through six years is shown above.

Treatment of ACH with r-hGH exaggerated the existing disproportion that is already quite significant, with very short limbs and relatively normal length backs. It is therefore our policy now to discuss and recommend future leg lengthening, in addition to growth hormone therapy. In this study, there was no significant dose effect and this may be important, as supra physiological doses of growth hormone advance skeletal maturity which is not ideal for optimal long-term height increments.

Analysis of group having completed 5 years of GH therapy (n=9), gave similar results and is shown below in table 8, expressed as mean  $\pm$  sem.

**Table 8 ACH: r-hGH Therapy**

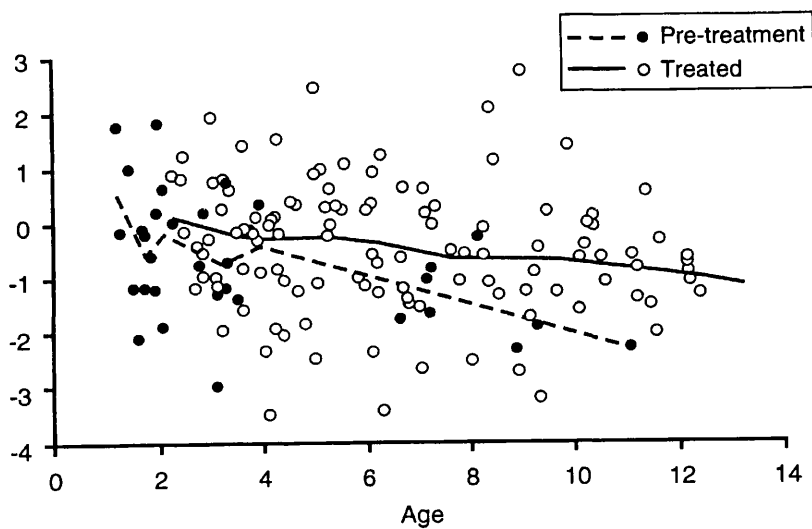
**DATA SET OF PATIENTS ON 5 YEARS OF THERAPY, N=9**

TREATMENT YEARS	HT SDS (mean $\pm$ sem)	SHSDS (mean $\pm$ sem)	SILLSDS (mean $\pm$ sem)
PRE TREATMENT	-5.0 (0.30)*	-1.1 (0.4)**	-7.85(0.4)
ONE YEAR	-4.23 (0.3)	-0.70 (0.5)	-6.90 (0.2)
TWO YEARS	-4.02 (0.3)	-0.23 (0.5)	-6.67 (0.2)
THREE YEARS	-3.74 (0.3)	0.53 (0.5)	-6.7 (0.2)
FOUR YEARS	-3.6 (0.3)	0.58 (0.4)	-6.37 (0.2)
FIVE YEARS	-3.5 (0.4)*	0.56 (0.4)**	-6.43 (0.26)

**Table 8** Progressive increase in \*Ht SDS (p <0.001) and \*\*SH SDS (<0.01) from baseline to year 5; SILL SDS increase from baseline to year 5 (p=0.01), but in comparison to SH SDS this was not significant, thus exaggerating the existing disproportion. No significant differences between year 4 and 5 in HT, SH and SILL SDS (p>0.01), although small number of patients.

**FIGURE 21**

**HV SDS Changes in Achondroplasia Before And During Treatment**  
**with r-hGH**



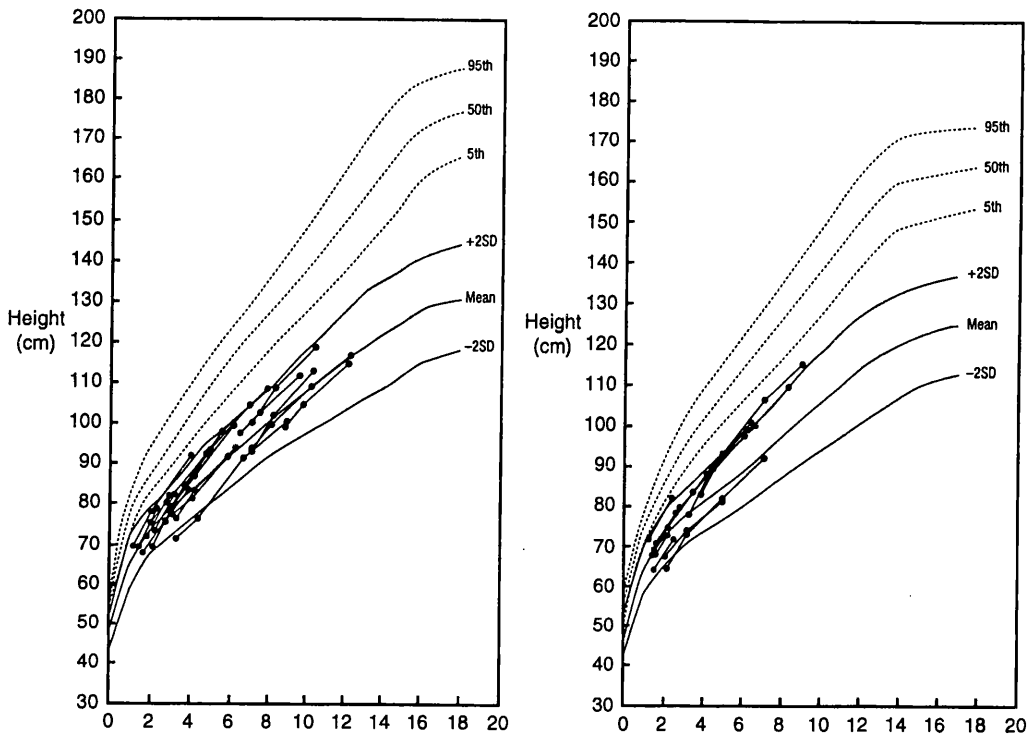
**Figure 21** HV SDS changes before and during therapy. Lowess locally weighted regression plot comparing treatment HV SDS data with similar data before treatment and according to age and time. For three patients, pretreatment HV SDS is not shown, as the pretreatment HV was calculated at other centres.

In the natural history of achondroplasia, it is known that after the first year of life there is a steady and progressive increase in height deficit. Growth hormone therapy, in the short term, prevented this accumulation of height deficit. This is demonstrated above in figure 21 of Lowess locally weighted regression plot.

The HV SDS, before treatment was started shows the expected steady decline in height velocity standard deviation scores and in contrast during treatment years the slope of the decline was significantly less steep. This was also seen when the height increments over the treatment years was plotted on disease specific achondroplasia growth charts (figure 22). The majority of our patients were quite young and a favourable response to r-hGH therapy over six years was noted. However, it is imperative as with other conditions in which growth hormone has been used (apart from true growth hormone deficiency, to analyse final height data prior to making any firm recommendations. It is encouraging to know that relatively few side effects to therapy have been described in the literature even with high doses of GH (KIGS data). In our cohort of patients one developed spinal stenosis at age 9, but this is a well know complication of ACH and was therefore not a definite complication related to the treatment with GH.

**FIGURE 22**

**Height Increments in Achondroplasia Over Time With r-hGH Therapy**



**Figure 22** shows height increment in ACH whilst on treatment with r-hGH. Individual linear heights of children with ACH during treatment with growth hormone were plotted on disease specific ACH growth charts. The ACH growth percentiles (solid lines) are shown together with Tanner's normal reference percentile (dotted lines). The left panel is the growth chart for boys and the right is for girls. Longitudinal height measurements plotted on these charts were continually maintained above the pretreatment percentiles.

#### **12.4 Mutation Positivity And Disproportion In Hypochondroplasia**

Analysis of the response to growth hormone in hypochondroplasia was not as straight forward as with achondroplasia and similar analysis was therefore not performed. This was due to the genetic and phenotypic heterogeneity and in addition the small numbers of HCH patients within the three groups based on disproportion scores described previously, made meaningful statistical analysis difficult. Therefore for analysis of response to growth hormone therapy the patients were divided into mutation positive and negative groups and comparisons were made with the results of children with Achondroplasia who were treated with growth hormone.

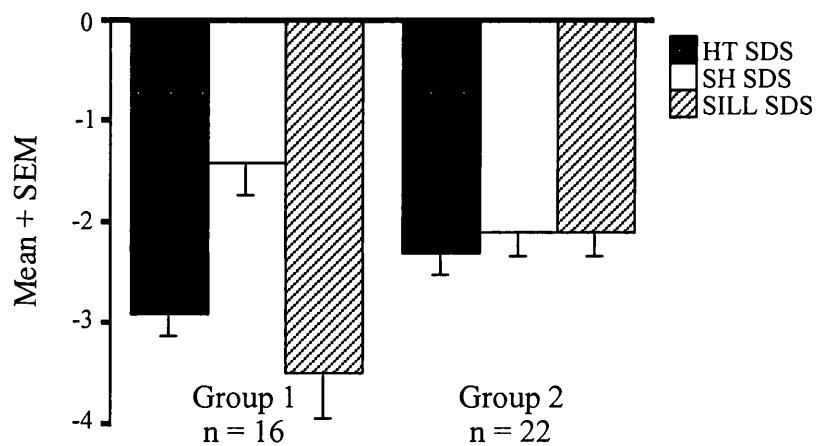
In this study there were 28/73 patients with the C1620A mutation. 45/73 patients were mutation negative. 16 mutation positive and 22 mutation negative patients were treated with r-hGH. The decision to treat depended on the pretreatment height velocity being less than  $-0.8$  SD. The baseline auxological presentation in mutation positive and negative groups is shown in figure 23.



**FIGURE 23**

**Baseline Auxology In C1620A Mutation Positive And C1620A Negative**

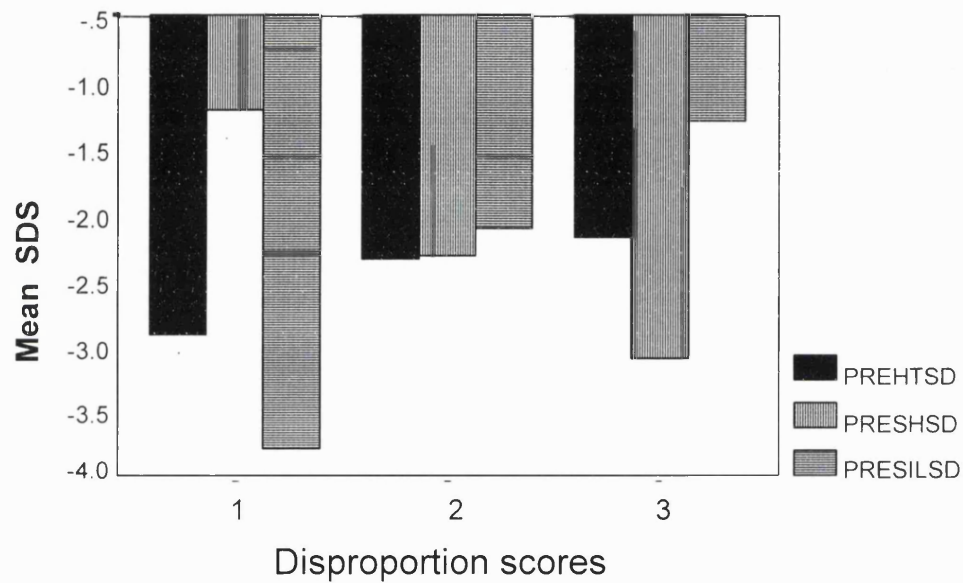
**Hypochondroplasia**



**Figure 23** demonstrates the baseline auxological measurements in the mutation positive and negative groups with the majority of the mutation positive patients being disproportionate with short legs.

**FIGURE 24**

**Disproportion Score In**  
**Hypochondroplasia**



**Figure 24** To further define the variable phenotype in HCH based on their body proportions, a disproportion score was derived. As mentioned previously, subtracting SILL SDS from SH SDS in each individual case derived this score. A score of 1 ( $\text{SHSDS} - \text{SILLSDS} \geq +1$ ) was obtained in those who had the classical presentation at the severe end of the disease spectrum with rhizomelic shortening, i.e. short limbs and normal or relatively spared spinal length. A score of 3 ( $\text{SHSDS} - \text{SILLSDS} \leq -1$ ) was obtained in those cases in whom the spinal length was significantly shorter than the limb length and score 2 ( $\text{SHSDS} - \text{SILLSDS} \leq +1$  to  $\geq -1$ ) was

obtained in those cases of HCH in whom there was relatively little disproportion and there was more or less an equal shortening of the back and limbs. Disproportion scores 1, 2 and 3 are demonstrated in figure 24 emphasizing the varied clinical spectrum of HCH. The number of patients in each group were as follows: DPS 1 = 19 patients, DPS 2 = 37 and DPS 3 = 15.

In two patients SH and SILL SDS was not available as the height data were obtained from out-reach clinics where body proportion measurements were not taken. Both patients however were not on r-hGH therapy.

The patient details and number of patients on GH therapy is shown in appendix 15.4

Clearly, the disproportion score demonstrated phenotypic heterogeneity within the group of HCH, despite all of the patients having the radiological criteria for inclusion, i.e. a lack of increase in interpedicular distance between L1 to L5. This phenotypic heterogeneity with respect to the disproportion, will have implications in screening for other mutations within FGFR3 and indeed raises the additional possibility, of other genes involved in this condition. Responses to growth hormone therapy in individual DPS groups was not possible due to small numbers of cases within each group. Therefore response to r-hGH therapy was analysed in the C1620A mutation positive and negative groups.

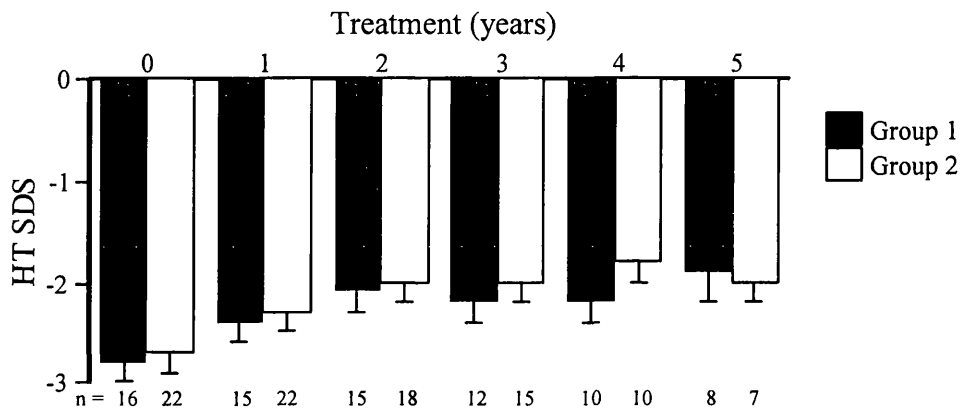
## 12.5 Growth Hormone Therapy

38/73 patients with HCH were on r-hGH therapy. Of this group, 16 were mutation positive. In our cohort of patients, 88% of those on r-hGH were prepubertal. r-hGH was administered at a median dose of 30U/m<sup>2</sup>/week (16-44 U/m<sup>2</sup>/week).

The response to r-hGH over a period of 5 years were analysed in both mutation positive and negative groups. There was a significant response to therapy in both groups as shown in figure 25. This was predominantly due to an increase in SH with relatively little increase in SILL in the mutation positive group as shown in figures 26 and 27. However, there was a more proportionate response in the mutation negative group (Group 1 Ht SDS F 9.0, p<0,01; SH SDS F 8.4, p<0.01; SILL SDS F 1.7,p Ns and Group 2 Ht SDS F 2.5, p=0.03, SH SDS F 4.07, p<0.01, SILL SDS F 0.44 ns). The response to r-hGH in the severe cases were therefore similar to that observed in our cohort of ACH.

**FIGURE 25**

**Height Increments With r-hGH Therapy In Hypochondroplasia**

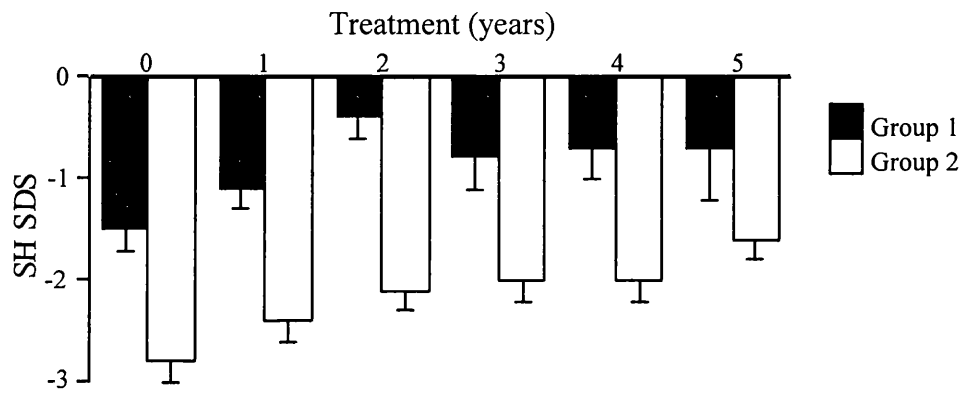


**Figure 25** demonstrates the 5 year response to r-hGH therapy in HCH in both C1620A mutation positive and negative groups. The response in both groups was significant.

Group 1 Ht SDS F 9.0,  $p < 0.01$ ; Group 2 Ht SDS F 2.5,  $p = 0.03$

**FIGURE 26a**

**SH SDS Increments With r-hGH Therapy In Hypochondroplasia**

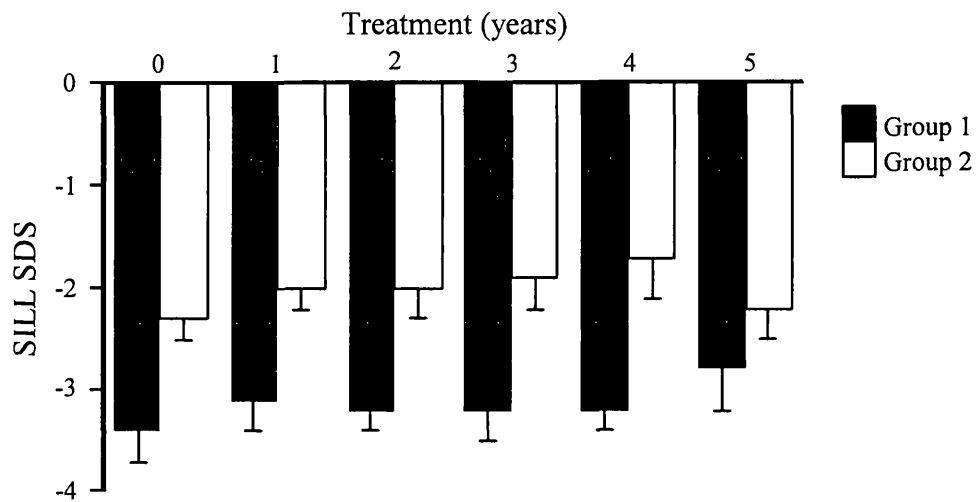


**Figure 26a** demonstrates the SH SDS increments with r-hGH therapy;

Group 1: SHSDS F 8.4,  $p < 0.01$ ; Group 2 SHSDS F 4.07,  $p < 0.01$

**FIGURE 26b**

**SILL SDS Increments With r-hGH Therapy In Hypochondroplasia**



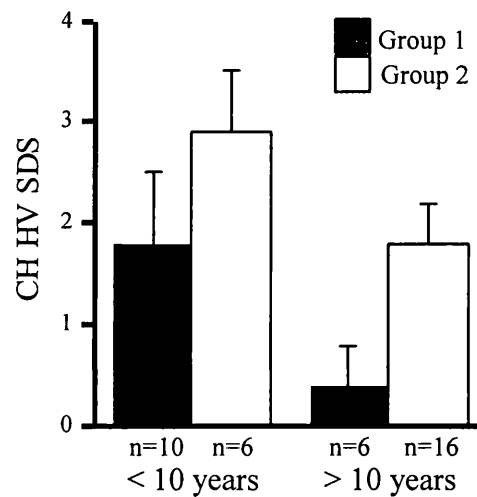
**Figure 26b** SILL SDS increments with r-hGH therapy in shown.

Group 1 SILL SDS F 1.7, p Ns; Group 2 SILL SDS F 1.4, p ns

**Figure 27**

**Response to r-hGH in Hypochondroplasia**

**Effect of Age and C1620A Mutation Positivity**



**Figure 27** demonstrates the first year response to growth hormone therapy in HCH depending on age (under 10 years and over 10 years of age at the start of therapy) and C1620A mutation positivity. The first year response to r-hGH expressed as a change in HV SDS was greater in the prepubertal patients (ANOVA F 7.1,  $p < 0.01$ ); first year response was greater in those children under 10 years of age and prepubertal.

The C1620A mutation positive cases appeared to do less well than the C1620A mutation negative cases, although this did not reach statistical significance (F 3.9,  $p = 0.06$ ).



The response to growth hormone was predominantly due to increases in sitting height in mutation positive patients, which exaggerated the existing disproportion. This was similar to the r-hGH response observed in patients with ACH.

In summary, the responses to r-hGH therapy in children with HCH were variable. Those who were prepubertal at the start of therapy and who were in addition, less disproportionate, appeared to do better than those within the severe end of disease severity. This is in fact not surprising, given the genetic and phenotypic variability amongst this group of children. As the majority in this study were prepubertal at the start of therapy, long-term follow up of these cases until final height is crucial to have definitive recommendations on treatment with growth hormone.

## **12.6 Limitations of Study and Justification of Study**

We fully recognise that this study has some limitations with respect to the study design. This was partly a retrospective study and analysis of longitudinal growth data of patients who were on r-hGH included auxological measurements obtained previously, in addition to ongoing data collected during my three year period as a research fellow.

The justification for this is as follows. All patients recruited in this study were patients attending the growth clinics at The Middlesex and Great Ormond Street Childrens Hospital. The auxological measurements were taken by accredited measurers in these growth clinics, which minimised the inter and intra observer variation. The rationale for starting growth hormone therapy in all these children was a poor growth velocity of less than  $-0.8$  SDS for a minimum of one year prior to starting r-hGH therapy. The numbers of patients recruited at the beginning of the study were small and this gradually increased over time. This explains the reason for small numbers having completed five years of treatment. In this study there were no dropouts during the six- year period. The demand from parents for r-hGH therapy was in fact increasing and we had to limit the numbers recruited into the study.

The criteria for the clinical diagnosis of ACH was straight forward with respect to the phenotype, which was supported by the radiological features

mentioned in chapter 7. However, in HCH, the clinical phenotype was more varied with some having the classical presentation of disproportionate short stature and others who were less disproportionate. Although it was clear that the disproportion was heterogenous and the Disproportion Score (DPS) confirmed this, r-hGH therapy in the individual DPS groups could not be reliably assessed due to small numbers in each group. All children with HCH and ACH, as mentioned previously, were significantly shorter when compared with their family heights. The invariable radiological feature in this group of patients was a lack of increase in interpedicular distance between L1 to L5. Radiological confirmation of diagnosis was made in all cases by experienced radiologists at the Middlesex Hospital and Great Ormond Street Childrens Hospital. This, in addition to height velocity, helped differentiate those children who were seen at our growth clinics, in particular the older children, who presented with constitutional delay of growth and puberty. However, I recognise that this criteria alone as a robust diagnosis of HCH must be taken into account with some caution when defining the HCH phenotype. Given the heterogeneity in the HCH phenotype, it is at present unclear, if in fact there is a subgroup that may have a different genotype. Therefore clearer definitions of the HCH phenotype, as shown by the disproportion score or genotype may in the future help in delineating the genotypic heterogeneity.

Lack of randomisation to r-hGH therapy in this study, is no doubt debatable and indeed a difficult situation. Treatment with growth hormone cannot be

easily randomised, given the nature of therapy with daily subcutaneous injections. In my view, albeit a very important issue crucial in clarifying the efficacy of therapy, administering placebo injections to children, particularly given the prolonged course of therapy, i.e. many years, would be far too traumatic to the child. It would be difficult to justify and obtain ethical approval. I was unable to find randomised trials of GH therapy in other conditions, and the reasons above probably hold true for other non growth hormone deficient disorders. I tried to overcome this problem, by comparing pre-treatment height velocities with year on year height velocity increments during r-hGH therapy. Although it may be argued that there will in any case, be an increase in first year height velocity in all children treated with GH, a sustained increase was noted in several patients with HCH and ACH, over a period of 5 years. This however, only reflects the short to medium term gain.

The dose of r-hGH used was variable, ranging from physiological replacement doses to supraphysiological doses of  $>25 \text{ U/m}^2/\text{week}$  and upto  $40 \text{ U/m}^2/\text{week}$  with a median dose of  $30 \text{ U/m}^2/\text{week}$ . This largely reflects personal practice in our department. The rationale for the higher dose of r-hGH was the possibility of growth hormone resistance in skeletal dysplasias, which has also been postulated in disorders including Turner Syndrome, with normal growth hormone secretion. The pre-treatment HVSDS in the high and low dose groups were similar ( $p>0.05$ ) in ACH. As mentioned previously, no obvious dose effects were noted in this study. In HCH, this was much more difficult due to the phenotypic heterogeneity and

the smaller numbers of patients. Despite the favourable medium term results of r-hGH therapy, in view of the wide dose range and varied response in HCH, final height data must be carefully analysed.

As with all growth disorders, final height data will eventually be the only robust and accurate data that will provide us with a true picture of the effectiveness of this treatment. Despite the lack of non-randomisation, I believe that this still forms an important study, with respect to the effectiveness of r-hGH therapy in HCH and ACH and more importantly the justification or otherwise of this expensive and difficult treatment. I would hope that my data would provide valuable information in assessing the final height of these children, who have been treated over many years with growth hormone therapy.

### 13. DISCUSSION

In 1994, the achondroplasia gene was mapped to the short arm of chromosome 4 and soon after, a single point mutation in FGFR3 (G1138A) was identified in virtually all patients with classical achondroplasia (Rousseau *et al.*, 1996 a). This then prompted the search for FGFR3 mutations in other skeletal dysplasias. A heterozygous mutation, C1620A, resulting in asparagine to lysine substitution at codon 540 (Asn540Lys) of FGFR3 was identified in about 40-50% of patients with HCH (Rousseau *et al.*, 1994; Bellus *et al.*, 1995 b; Rousseau *et al.*, 1996 b; Bonaventure *et al.*, 1996 a; Bonaventure *et al.*, 1996 b). The reason for the high frequency of the G1138A mutation in ACH is unknown, although it has been hypothesised that the presence of a (CpG) dinucleotide in codon 380 resulting in G1138A transition, is a hot spot for mutations in ACH and may affect a residue critical for signal transduction. It has been shown that a high frequency of polymorphisms are present in DNA sequences containing CpG dinucleotides (Barker *et al.* 1984). The methylated cytosine in these CpG sequences can spontaneously deaminate and this results in a substitution of cytosine by thymidine and therefore a substitution of guanine by adenine on the opposite sense strand. Mutation rates in these CpG dinucleotide sequences have been estimated to occur at a rate of  $3.68 \times 10^{-8}$  to  $1.05 \times 10^{-7}$ .per gamete per generation (Bellus *et al.*, 1995). This is almost 50 to 760 times greater than the previously estimated rate of the G1138A mutation of FGFR3, which had been calculated

according to the prevalence of achondroplasia and had been estimated to be around 1 in 15,000 to 1 in 77,000 (Oberklaid *et al* 1979., Gardner 1977).

Bellus *et al* (1995 b) also demonstrated an increase in the rate of transversion mutations in FGFR3 by 1700 to 3300 times in comparison to previous studies (Koeberl *et al.*, 1973). 2.6% of patients in his study had G-C transversion (G1138C transversion) and hence an increase in the mutation rate from  $4.1 \times 10^{-10}$  to  $6.96 \times 10^{-7}$  per generation per gamete. Although the exact reason for this increased mutation rate is unknown, one explanation could be the presence of a CTT trinucleotide sequence about 8-11 base pairs downstream of nucleotide 1178 which may increase the incidence of point mutations adjacent to it (Bellus *et al.*, 1995 a). On the basis of estimates of the prevalence of achondroplasia, the mutation rate in FGFR3 1138 guanosine nucleotide is two to three orders of magnitude higher than that previously reported for transversions and transitions in CpG dinucleotides. To date, this represents the most mutable single nucleotide reported in the human genome. In the presence of a high prevalence of G1138A and G1138C mutation in the transmembrane domain of FGFR3 resulting in achondroplasia with phenotypic homogeneity, it could only be assumed that apart from functioning as a membrane anchor, the transmembrane domain may also have an important role to play in signal transduction. It had also been hypothesised that there may be a FGFR3 pseudogene (Bellus *et al.*, 1995 a) that may result in gene conversions, which have been shown to cause recurrent mutations in other diseases like congenital adrenal

hyperplasia due to 21 hydroxylase deficiency. However, there have been no reports of a FGFR3 pseudogene as yet, although I wondered if this might explain the PCR doublet in my samples described previously.

The relatively easy and reliable method of identification of these mutations using restriction enzyme digestion of amplified DNA enables accurate prenatal diagnosis (Bellus *et al.*, 1994) but this may only be relevant to avoid lethal homozygous achondroplasia. The offspring of 2 parents with heterozygous achondroplasia will have a 25% chance of homozygosity and 50% chance of developing heterozygous achondroplasia with only 25% chance of being homozygous normal.

The international Working Group on Constitutional Disease of Bone (1992) have classified osteochondrodysplasias according to specific radiodiagnostic criteria and although it is far from being complete, it provides a comprehensive list of skeletal dysplasias and where available, information on the mode of inheritance, gene localisation and the encoded defective protein are given. This list was first published in 1992 and did not include FGFR3 mutations causing Achondroplasia, which was first reported in 1994. It is interesting to note that other osteochondrodysplasias classified under the same group of defects of the tubular (and flat) bones and/or axial skeleton including Hypochondroplasia and Thanatophoric Dysplasia have also been shown to result from missense mutations in FGFR3, further



emphasizing the important role of FGFR3 in embryogenesis especially in relation to the developing limb bud.

FGFR mutations have now been associated with various skeletal dysplasias and craniofacial syndromes. Pfeiffer syndrome, an autosomal dominant craniofacial syndrome with craniosynostosis, syndactyly and deviation of the thumbs and great toes (Rutland *et al.*, 1995; Muenke *et al.*, 1995; Lajeunie *et al.* 1995), results from mutations occurring in both FGFR1 and FGFR2 and hence demonstrates that this is a heterogenous condition. FGFR2 mutations are also described in other craniofacial syndromes associated with craniosynostosis including Apert syndrome, Crouzon syndrome and Jackson-Weiss syndrome, all of which have distinctive phenotypes. However, allelic heterogeneity has been described with these syndromes (Rutland *et al.*, 1995; Muenke *et al.*, 1994; Lajeunie *et al.*, 1995; Jabs *et al.*, 1994; Wilkie *et al.*, 1995 b; Reardon *et al.*, 1994).

Mutations in the extracellular ligand binding domain and intracellular tyrosine kinase domains of FGFR3 resulting in Thanatophoric Dysplasia, a lethal skeletal dysplasia sharing several phenotypic characteristics with homozygous Achondroplasia has also been recently described (Tavormina *et al.*, 1995). The FGFR3 expression in growth plates has been shown to be much higher than that of FGFR1 and FGFR2 (Partanen *et al.*, 1991) and therefore it is not surprising that mutations in FGFR3 result in skeletal dysplasias which result in severe shortening of the limbs in comparison to

the craniofacial syndromes. No skeletal disorder has yet been linked to FGFR4.

More recently, a unique phenotype of severe achondroplasia with developmental delay and acanthosis nigricans has been described (Tavormina et al., 1999; Bellus *et al.*, 1999) and abbreviated as SADDAN. A Lys650Met in the distal tyrosine kinase domain of FGFR3, adjacent to the mutation responsible for Thanatophoric Dysplasia type I (TDI) has been described in this group of cases. This mutation has been shown to constitutively activate FGFR3 at least two to three times greater than that seen with the mutation resulting in TDI. Histological studies have shown severe abnormalities in enchondral bone growth. The cause for the developmental delay and the acanthosis nigricans seen in this condition that is not present in other skeletal dysplasias with similar point mutations in FGFR3, is not known.

Hypochondroplasia is a disorder with a wide spectrum of disease severity. It is a heterogeneous disease with patients at the severe end of the spectrum presenting with disproportionate short stature and those with a milder disease present with short stature and a lack of a pubertal growth spurt. In this study, there was a wide phenotypic spectrum of disease severity and as seen with other studies worldwide, only 50% or less of these patients have mutations in FGFR3. Further linkage analysis and better molecular characterisation of the mutation negative patients is needed to define this

condition with clarity. Further genetic characterisation of this diverse group is required to define the condition more effectively. Multicentre studies are required for further genetic studies including linkage analysis.

73 children presenting to our clinics with short stature and radiological evidence of HCH were screened for this mutation and was identified in 38% of cases. In our study, children with the C1620A mutation had a severe phenotype with patients presenting with disproportionate short stature. Those with a milder phenotype were C1620A mutation negative not obviously disproportionate. In addition to the genetic and phenotypic heterogeneity in HCH, the response to growth hormone therapy was also variable. ACH phenotype and genotype, on the other hand, was more homogenous as was the response to r-hGH.

Although it has been the view of our department to consider the milder end of the HCH disease spectrum to be underdiagnosed, care must be taken whilst considering this diagnosis. It may be argued that those in group 2 of the disproportion score (DPS 2), presenting with proportionate short stature were in fact short normal. One needs to mention that in my study, the invariable radiological finding that lead to the diagnosis of HCH in addition to other radiological, auxological and clinical characteristics depending on the disease severity, was the absence of increase in interpedicular distance between lumbar vertebrae L1 to L5. This radiological finding was absent in those presenting to the growth clinic who

were diagnosed to have familial short stature or constitutional growth delay. However, in my view, it is not entirely clear if a similar radiological finding of the lumbar spine will be observed in other causes of short stature. The limitations of this study have already been described at length in chapter 12.

We were able to subdivide our cohort of HCH into two groups depending on the mutation analysis. The first group with the C1620A mutation were more severely affected, with disproportionate short stature resembling ACH. The second group were negative for this common mutation, had a milder phenotype presenting with short stature with almost equal reduction in both SH and SILL. SSCP analysis of the transmembrane and tyrosine kinase domains of FGFR3 failed to identify any significant sequence variation in these individuals. These negative results neither confirm nor exclude mutations in FGFR3 as the cause of disease. In previously described studies, the common mutation had been identified in only 50% of patients with clinically proven HCH and no other mutations were found despite screening more than 90% of the coding sequence (Bellus *et al.*, 1995 b; Prinos *et al.*, 1995). Therefore, there remains the possibility that either mutations are present outside the coding sequence or other genes may be involved. In our study the majority of cases were sporadic, with only 6% of cases with one affected parent and it was therefore not possible to establish linkage to chromosome 4 by other means.

The mutations described so far in several genetic forms of short stature have been mapped to a few exons in the FGFR3 encoding the tyrosine kinase and transmembrane domains, suggesting a significant correlation between abnormal signaling via the mutant receptors and the phenotype. These FGFR3 mutations have a gain of function, demonstrated by constitutive activation of the mutant receptor in the absence of ligand (Webster *et al.*, 1996; Wilkie *et al.*, 1995 a; Chen *et al.*, 1997). This suggests that excessive activation of FGFR3 results in inhibition of cell growth in cartilaginous growth plates. More recently, it has been shown that the mutant FGFR3 in Thanatophoric Dysplasia type II resulted in activation of the Stat1 signaling pathway, a negative regulator of cellular growth. The severity of the disease appears to be directly proportional to the degree of activation of the signaling pathway (Webster *et al.*, 1996; Webster *et al.*, 1997; Chen *et al.*, 1997; DiLeone *et al.*, 1997). The G1138A and C1620A mutations of FGFR3 resulting in ACH and HCH were shown to activate Stat 1 to varying degrees (Wu-Chou *et al.*, 1997).

Receptor specificity plays an important role in governing the activity of the fibroblast growth factors. It has been shown that specific fibroblast growth factor ligands interact in a very specific manner with the fibroblast growth factor receptors. This has significant implications for specific growth patterning in the developing limb and craniofacial structures in the mouse embryo (Santos Ocampo *et al.*, 1996; Hecht *et al.*, 1995 a; MacArthur *et al.*, 1995; Mathieu *et al.*, 1995).

Little is known about the pathophysiology of the defect in HCH although the severe variant resembles ACH, with defective endochondral ossification of long bones. Naski *et al* (1998) studied the expression of FGFR3 in proliferating chondrocytes in growth plate cartilage. Their aim was to study the mechanism by which FGFR3 mutations result in inhibition of endochondral ossification of growth plate cartilage. The expression of activated growth plate cartilage in mice were targeted using regulatory elements from type II collagen gene. FGFR3 stimulation was shown to inhibit endochondral bone growth by significantly inhibiting chondrocyte proliferation and by slowing chondrocyte differentiation. They also studied the effect of this on signaling pathways BMP4 and Indian Hedgehog. These signaling pathways were shown to be significantly down regulated in growth plate chondrocytes and in the perichondrium. Conversely in the FGFR3 deficient mice, BMP4 expression was up regulated in the growth plate chondrocytes suggesting that FGFR3 is an upstream negative regulator of certain signaling pathways involved in proliferation and differentiation of growth plate chondrocytes (Naski *et al.*, 1998).

Homozygous mutated transgenic mice with a Lys644Glu substitution in FGFR3 demonstrated retarded endochondral bone growth, with the severity being directly related to the level of expression of the mutated FGFR3. The mutated FGFR3 was also shown to result in activation of Stat 1, Stat 5a, Stat5b and in up regulation of cell cycle inhibitors p16, p18 and p19. The

mutant growth plates as a result were in a less active state and had fewer numbers of proliferating chondrocytes in growth plates (Li *et al.*, 1999).

Recently, a mouse model for ACH has been developed and this will enable further studies in understanding the signaling pathways of FGFR3 (Wang *et al.*, 1999). Gene targeting and introduction of the ACH common mutation into the murine FGFR3 was performed (Wang *et al.*, 1999). Insertion of the ACH mutation resulted in the dominant achondroplasia dwarf with decreased size, midface hypoplasia, distorted foramen magnum, kyphosis and defective growth plates of the long bones. This demonstrated that achondroplasia occurs as a result of gain of function of FGFR3 and inhibition of chondrocyte proliferation (Wang *et al.*, 1999). Furthermore, Deng *et al.*, 1996, had previously showed the role of FGFR3 as a negative regulator of bone growth. Disrupting the FGFR3 gene in mice was shown to cause prolonged bone growth. This growth was shown to be accompanied by expansion of proliferating and hypertrophied chondrocytes within the developing growth plates. It was therefore concluded that FGFR3 regulated enchondral ossification by a negative regulation and thus reduced osteogenesis rather than promoting it. In ACH and HCH, mutations in FGFR3 are likely to have a gain of function that activates negative growth control and therefore results in stunted growth.

Further studies of the extremely complex fibroblast growth factor signaling pathway with its multiple ligands and four receptors may provide important

answers about the role of these receptors in organogenesis and bone development.

The clinical variability seen in HCH has been previously described (Wynne Davies *et al.*, 1981). The emphasis of disproportionate short stature as the major clinical finding in HCH holds true for the severe end of the spectrum. However most of these children grow slowly, are less obviously disproportionate and have a reduced pubertal growth spurt resulting in significant reduction in final height in relation to their height prediction. It is important to be aware of this group of children as growth hormone therapy has been shown to restore the pubertal growth spurt (Appan *et al.*, 1990). It is also important to emphasise that the characteristic radiological feature of decreased interpedicular distance between lumbar vertebrae L1 to L5 with short pedicles in the absence of other significant radiological abnormalities is an invariable feature, regardless of the severity in phenotype. The genetic basis of the milder phenotype is yet undetermined. The identification of the C1620A mutation positive individuals as a separate cohort of hypochondroplasia may allow for better comparison of growth data and response to management in what is a heterogenous disorder. This makes follow up of these children to final height very important to obtain meaningful and robust answers with respect to the natural history and treatment responses.



Growth hormone is an important regulator of linear growth. The linear skeletal growth is dependent on the proliferation and maturation of chondrocytes and endochondral ossification of the growth plate cartilage. The natural history of severe Hypochondroplasia is a gradual decrease in height velocity when compared to normal standards (Appan *et al.*, 1990; Bridges *et al.*, 1991) and those with a milder phenotype appear to grow normally until puberty when the pubertal growth spurt is attenuated. Since the introduction of recombinant human growth hormone, the wider indications for growth hormone therapy in short stature conditions not associated with growth hormone insufficiency has attracted increasing interest.

ACH is one of the most severe forms of skeletal dysplasia with individuals on average being 50cms shorter than the normal adult population. The nature of the growth problem in such conditions is most likely due to reduced sensitivity to the action of GH and IGF-s (Zadik *et al.*, 1992). Hence supraphysiological GH therapy has been used in an attempt to overcome skeletal resistance. Undoubted initial short term benefits in both ACH and other skeletal dysplasias including Turners syndrome (Hindmarsh *et al.*, 1991; Bridges *et al.*, 1994; Darendeliler *et al.*, 1990a, b) have been described, although not all have been translated into significant increments in predicted adult height (Rosenfeld *et al.*, 1998; Taback *et al.*, 1996). Differences in age at onset of therapy, duration and dose of therapy, method and accuracy of height prediction as well as a secular trend to increased

adult height (Breunen *et al.*, 1980; Freeman *et al.*, 1995) may have all contributed to these discrepancies.

We report a significant benefit on longitudinal measurements of Ht SDS in an uncontrolled intervention study of 35 cases of ACH, but our data was strengthened by a larger number of a mostly prepubertal cohort of a much younger age (2.25 years) and longer treatment duration (up to 6 years). In addition, we have been able to assess the effects of a wide range of r-hGH doses on the response. We were not able to show a significant dose response relationship even in the first year of treatment and this was surprising given the experience in other related conditions (Hindmarsh *et al.*, 1991; Bridges *et al.*, 1994). However, beyond the first year of life, higher doses have been shown to advance skeletal maturity and shorten the duration of puberty (Darendeliler *et al.*, 1990 b) thereby limiting the eventual growth potential. Thus it will be important to compare outcomes of low versus high dose therapy at adult height, before making recommendations on the dosage at therapy.

In the natural history of ACH, there is a progressive accumulation of height deficit. After the first year of life, the growth rate in untreated children approximates only the third velocity centile of normal children and remains at this slow rate for the rest of childhood (Horton *et al.*, 1978; Horton *et al.*, 1977). In comparison with the growth rates of normal children, which oscillate about the 50th centile, this is clearly abnormal. Early r-hGH

therapy in children with Achondroplasia prevented the accumulating height deficit by maintaining growth velocity near to the normal range and maximising the potential of normal growth in the spine, at least in the medium term. Final height data are crucial to see if this response is sustained. Although subischial leg length remained significantly compromised, no further deficit was incurred over the treatment period. The accentuation of the existing disproportion in ACH due to the variable SH and SILL responses to r-hGH therapy that was demonstrated in this study has not been reported and in most cases not examined in previous studies of r-hGH in ACH (Hindmarsh *et al.*, 1991; Horton *et al.*, 1992; Nishi *et al.*, 1993; Yamate *et al.*, 1993; Bridges *et al.*, 1994; Shohat *et al.*, 1996; Weber *et al.*, 1996; Stamoyannou *et al.*, 1997; Tanaka *et al.*, 1998; Seino *et al.*, 1999). The greater number of patients having a longer duration of therapy in our study may account for this accentuation of disproportion being demonstrated.

The abnormal bones in ACH and HCH make estimates of skeletal maturity unreliable. The inter and intraobserver error even in the normal situation is well documented (Breunen *et al.*, 1980), and is increased in children with skeletal dysplasias. We have therefore not used this as a method of predicting final height. Instead, we have used growth charts available from historical ACH patients for comparison (Horton *et al.*, 1978). Such a method has been used to assess the treatment effects in other conditions (Lyon *et al.*, 1985) and carries with it the potential of misrepresenting final

outcomes if there has been a significant secular trend. We demonstrated that our subjects were comparable to historical controls at the time of onset of treatment whilst the magnitude of the secular trend is unlikely to significantly affect the reported outcome. The magnitude of growth improvement documented in ACH, was an increase of one Ht SDS over 6 years, an increment of 8cms in terms of “height gained”. Nevertheless the individual responses reported were very variable.

Short to medium term gains in height SDS has been demonstrated in HCH. Previous studies have shown favourable responses in those presenting in later childhood with a loss of pubertal growth spurt (Bridges *et al.*, 1991; Bridges *et al.*, 1994; Appan *et al.*, 1990). In this study, the response to growth hormone was variable. A better response, in terms of increment in Ht SDS was demonstrated in children under the age of 10 years. Treatment of ACH and HCH with r-hGH must only be carried out as part of a clinical trial, until such time that firm recommendations on its efficacy can be made, which further emphasis yet again on the importance of final adult height data in this cohort. Although our patients were strictly speaking within a trial with growth hormone prescriptions on a named patient basis only, the doses of r-hGH used reflected personal experience and practice of the head of our endocrine department with a median dose of 30U/m<sup>2</sup>/week. The rationale for the higher dose of r-hGH was the possibility of growth hormone resistance in skeletal dysplasias, which has also been postulated in

disorders including Turner Syndrome, with normal growth hormone secretion.

The pretreatment height velocity in the C1620A mutation positive and negative groups was similar. The majority of these patients were prepubertal and all had age appropriate puberty. The response to treatment over 4 years was measured by an increase in height SDS and height velocity SDS in both groups. This response to r-hGH was greater in the C1620A mutation negative group. The increase in body proportion was also different in the two groups. There was a significant increase in sitting height in comparison to the leg length in the mutation positive group, which accentuated the existing disproportion, as observed in ACH. The mutation negative group demonstrated a proportionate increase in height with increases in both sitting height and subischial leg lengths. Follow up of these patients to final height is necessary to assess the long-term effects of r-hGH therapy in HCH. As seen in ACH, there was no significant dose effect seen in HCH treated with growth hormone. The effects of r-hGH on final height are not known, but GH therapy, coupled with the opportunity of leg lengthening, will alleviate the disproportion and carry the possibility of adult stature for these patients within the lower end of the normal range. The dose of r-hGH and timing(s) of surgical intervention(s) needs to be established, once adult height data are confirmed.

## 14. CONCLUSIONS

ACH and HCH are genetic disorders of disproportionate short stature. ACH being at the severe end of the spectrum and HCH presenting with a varied clinical spectrum. At the severe end of the spectrum hypochondroplastic patients resembled ACH with rhizomelic short stature (short limbs and normal backs), although the majority of the cases were not as severe as those with ACH. At the milder end of the spectrum, there was a wide degree of severity of disproportionate short stature, with some not presenting until later with a pubertal growth arrest. In achondroplasia, the phenotype and radiological features were homogenous with little variability.

Mutations in the transmembrane domain of FGFR3 have been identified in the majority of the patients with ACH. In contrast, only about a third to half of hypochondroplastic patients at the severe end of the spectrum have mutations in the tyrosine kinase domain of FGFR3. The underlying genetic cause of the remaining group of patients with hypochondroplasia remains unknown. Large collaborative studies are needed to study and understand the genetics of this group of patients.

Mutations in both the transmembrane domain and tyrosine kinase domain constitutively activate the receptor. The severity of the disease is dependent on the degree of ligand independent activation of the fibroblast growth factor receptor 3 gene.

The inheritance pattern in these conditions is autosomal dominant but the majority are due to sporadic mutations. In our study the number of familial cases were too few to perform meaningful linkage analysis and this further strengthens the need for future collaborative studies.

All patients with hypochondroplasia were significantly short in comparison to their family. Those with the C1620A mutation were severely affected with rhizomelic short stature. The mutation negative patients were less severely affected and were much less disproportionate short stature. The phenotype was further subdivided depending on the nature of the disproportion. At the severe end of the spectrum, were those with short limbs and normal backs. At the milder end of the spectrum, they presented with near proportionate short stature and there were some children with a reversal of disproportion with shorter backs. The relevance of this is yet unclear, although it clearly emphasizes the need for further characterization of the genotype in this subgroup.

In this study regardless of the severity of the disproportion, all patients with hypochondroplasia had the invariable radiological characteristic absence of increase in interpedicular distance between lumbar vertebrae L1 to L5. This differentiated those patients that were seen in the growth clinics with constitutional delay of growth and puberty and familial short stature. However, in my view, it is at present unclear if a similar radiological finding

will be observed if many more children with other causes of short stature are examined.

The response to growth hormone therapy in achondroplasia, up to a period of six years was favorable, with significant and steady increase in Ht SDS over the six year period. The most significant finding in this group, was that during treatment with growth hormone there was prevention of accumulation of height deficit during those treatment years. The response was also better in those who were much younger (median age of 2.25 years) which would result in the accumulated height deficit being much less at the start of growth hormone therapy. The height increments were predominantly due to increases in the length of the back and this exaggerated the existing disproportion.

As with the genetic and phenotypic heterogeneity, the responses to growth hormone therapy in HCH were also quite variable. In this study the responses to treatment with GH was better in the younger patients and unlike achondroplasia patients the treatment response was better in the mutation negative patients. Until final height is achieved in this cohort of cases, firm recommendations on treatment with growth hormone therapy cannot be made.

With the knowledge of the disappointing response to r-hGH in the short normal children (Hindmarsh et al., 1996) it may be argued that growth



hormone in skeletal dysplasias is not justified. However it is becoming clear that in some skeletal dysplasias including achondroplasia and severe hypochondroplasia, r-hGH, at least in the medium term maintains the height velocity within the normal range thus reducing the height deficit incurred over time. Supra physiological doses of growth hormone have not been shown to be beneficial and this is especially important to bear in mind with respect to the cost of r-hGH and potential for adverse effects in the long term. The limitations in this study with respect to retrospective data, non randomisation and wide range of growth hormone dose has been eluded to on several occasions and in addition detailed in section 12.6.

Despite the lack of non-randomisation, I believe that this still forms an important study, with respect to the effectiveness of r-hGH therapy in HCH and ACH and more importantly the justification or otherwise of this expensive and difficult treatment.

A combined treatment with growth promoting agents and leg lengthening in the severe cases with obvious disproportion in both achondroplasia and hypochondroplasia may provide these children with final heights at the lower end of the normal range. It continues to be important to emphasise that this expensive therapy needs to be carefully evaluated with analysis of growth data of these children to final height. In addition clear guidelines to surgical leg lengthening are needed.

## 15. APPENDICES

### 15.1 FGFR3 cDNA Sequence (accession no M50581)

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1  cgcgcgctgctgaggacgcgcgccccgccccgcccgcctgcccgcctgctgccc
61  ctgcgctctgctgcccgtggccatcgtggccggcctcctcggagtccttggggagc
121  gagcagcgcgctcgtggggcagcggcagaagtcccggcccagagcccggccagcaggag
181  cagttggctctcggcagcgggatgctgtggagctgagctgtccccgcccgggggtggt
241  cccatggggcccactgtctgggtcaaggatggcacagggtggtgcccctcggagcgtgtc
301  ctgggtggggcccagcggctcaggtgctgaatgctcccacgaggactccggggcctac
361  agctgcccggcagcggctcacgcagcgcgactgtgcccacttcagtgctgcccgtgacagac
421  gctccatcctcgggagatgacgaagacggggaggacaggctgaggacacaggtgtggac
481  acaggggccccctactggacacggcccagcggatggacaagaagctgctggccgtgccc
541  gccgccaacaccgtccgcttccgctgccagcgcgctggcaaccccactccctccatctcc
601  tggctgaagaacggcagggagtcccgcgagcaccgcatggaggcatcaagctgccc
661  catcagcagtgaggccggtcatggaaagcgtggtgcccctcggaccgcccgaactacac
721  tgcgctgctggagaacaagtgtggcagcatccggcagacgtacacgctggacgtgctggag
781  cgctccccgcaaccgcccacatcctgcaggcgggctgcccggccaaccagacggcggctg
841  ggcagcagcgtggagtccactgcaagggtacagtgacgcacagcccacatccagtg
901  ctcaagcagctggaggatgaacggcagcaagggtggcccggcagcgcacaccctacgttacc
961  gtgctcaagacggcggcgctaacaccaccgacaaggagctagaggttctctccttgcac
1021  aacgtcaccttggagcgcggggagtacacctgctggcgggcaattctatggggtt
1081  tctcatcactctgctggctggtggtgctgccagccgaggaggagctggtggaggctgac
1141  gaggcggcagtgatgacagcctcctcagctacggggtgggcttcttctgttcatc
1201  ctggtggtggcggctgacgctctgcccgcctgcccagcccccccaagaaagcctgggc
1261  tccccaccgtgcacaagatctcccgttcccgtcagagcagaggtgtccctggagtcc
1321  aacgcgtccatgagctccaacacaccactggtgcccacgcagaggtgtcctcaggggag
1381  ggccccacgctggccaatgtctccgagctcgagctgcccagcccccaaatgggagctg
1441  tctcggcccggctgaccctgggcaagcccctggggagggctgctcggccaggtggtc
1501  atggcggaggccatcggcattgacaaggaccggcccgaagcctgtcaccgtagcccgtg
1561  aagatgctgaaagacgatgccactgacaaggacctgcccagcctggtgctgagatggag
1621  atgatgaagatgatcgggaaacacaaaaacatcatcaacctgctggcgcctgcacgcag
1681  ggcgggccccctgtacgtgctggtggagtacgcggccaagggtaacctgcgggagtttctg
1741  cgggcgcggcggcccccgccctggactactccttcgacacctgcaagcccggcaggag
1801  cagctcacctcaaggacctggtgctcctgtgctaccaggtggcccggggcatggagtac
1861  ttggcctcccagaagtgcacccagggacctggctgcccgaatgtgctggtgaccgag
1921  gacaacgtgatgaagatcgcagacttcgggctggcccgggacgtgcacaacctcgactac
1981  tacaagaagacaaccaacggcggctgcccgtgaagtggatggcgcctgagcccttgttt
2041  gaccgagctcactcaccagagtgacgtctggtccttggggctcctgctctgggagatc
2101  ttacgctgggggctccccgtacccggcatccctgtggaggagctctcaagctgctg
2161  aaggaggccaccgcatggacaagcccgccaactgcacacagacctgtacatgatcatg
2221  cgggagtgctggcatcccgcgcctcccagaggcccacctcaagcagctggtggaggac
2281  ctggaccgtgctccttaccgtgacgtccaccgacgagtacctggacctgtcggcgccttc
2341  gagcagctactccccgggtggccaggacccccagctccagctcctcaggggacgactcc
2401  gtgtttgcccacgacctgctgccccggccccaccagcagtgggggctcgcggacgtga
2461  agggccactggtccccacaatgtgaggggtccctagcagcctcctgctgctggtgca
```

## **15.2 Tyrosine Kinase Domain Of FGFR3**

**( IIIb variant of FGFR3; Keegan et al 1991)**

ctgagagtggcgagttttcacactcatggtccctctgcctccactgccaGGCTGACCCTGGGC  
AAGCCCCTTGGGGAGGGCTGCTTCGGCCAGGTGGTCATGGCGGA  
GGCCATCGGCATTGACAAGGACCGGGCCGCAAGCCTGTCACCG  
TAGCCGTGAAGATGCTGAAAAGgtgaggagggggcgccaggggtgcagagcagggc  
tgggggcccgcgcccgcctgacacaggccccccgctcctgcacagACGATGCCACTGACA  
AGGACCTGTCGGACCTGGTGTCTGAGATGGAGATGATGAAGATG  
ATCGGGAAACACAAAAACATCATCAACCTGCTGGGCGCCTGCAC  
GCAGGGCGtaggtgcggtagcggcggtggtgccggctgggcgccctcctgggcctggcagccc  
gtctgaggagcccgtgtcccaggGCCCCTGTACGTGCTGGTGGAGTACGCGG  
CCAAGGGTAACCTGCGGGAGTTTCTGCGGGCGCGGCGGCCCCCG  
GGCCTGGACTACTCCTTCGACACCTGCAA

### 15.3 Primer Sequences

Uppercase: exonic sequence; lower case intronic sequence; Genbank

accession: M58051; F/R: forward or reverse sequence; code: primer ID.

<b>primer sequence</b>	<b>location</b> <b>m58051</b>	<b>Nucl.</b>	<b>F/R</b>	<b>code</b>	<b>conc.</b>
5' ctgagagtgggcgagttttcac	intronic	22	F	SO295	122 uM
5' TTGCAGGTGTCTCGAAGGAGTAGTC	1787-1765	23	R	SO296	96uM
5' cagccctgctctgcaccct	intronic	20	R	HCH-R1	128uM
5' ATGCTGAAAGgtgaggagg	intron/exon	20	F	HCH-F2	72uM
5' ctctcagacgggctgccagg	intronic	21	R	HCH-R2	124uM
5' tagcggcgggtgcccggct	intronic	20	F	HCH-F3	130uM
5'aggagctggtggaggctga	1121-1139	19	F	SO293	110uM
5'GGAGATCTTGTGCACGGTGGGG	1284-1262	22	R	SO294	108 uM
5' GGGAAACACAAAAACATCATCAAC		25		T3729N	117uM
5' GGGAAACACAAAAACATCATCAA		25		T3730M	67uM
5' CATCAATCAACCTGCTGGG	1750-1767	19		SO297	92uM
5' CATCAATCAACCTGATGGG	1659A	19		SO298	84uM
5' ctgagagtgggcgagttttcac Texas red				SO295-TR	10.7 uM

Nucl. - nucleotide; Conc. - concentration

### 15.4 Patient Details

**A: growth hormone therapy: yes = 1, no = 2; B: common mutation:**

**positive =1 and negative = 2; C: disproportion score (DPS) 1=DPS >1 ;**

**2= DPS <1>-1; 3= DPS <-1; D : growth hormone dose in U/m<sup>2</sup>/week.**

No	Name	A	B	C	D	No	Name	A	B	C	D
1	A W	1	1	1	25	20	C W	2	1	1	.
2	C A	1	1	1	30	21	D W	2	1	2	.
3	C R	1	1	2	30	22	EB	2	1	.	.
4	J R	1	1	1	40	23	J B	2	1	2	.
5	J E	1	1	2	30	24	J W	2	1	2	.
6	J H	1	1	1	25	25	J E	2	1	1	.
7	J P	1	1	3	30	26	L W	2	1	1	.
8	L T	1	1	1	30	27	R A	2	1	2	.
9	M S	1	1	1	30	28	W T	2	1	1	.
10	M A	1	1	1	30	29	B C	1	2	2	30
11	T H	1	1	1	20	30	B L	1	2	2	30
12	R B	1	1	1	30	31	C S	1	2	3	.
13	R C	1	1	1	25	32	C B	1	2	2	40
14	R W	1	1	2	30	33	C M	1	2	3	20
15	S C	1	1	1	30	34	D L	1	2	2	30
16	S N	1	1	2	30	35	D A	1	2	2	30
17	OPP	1	1	2	20	36	D M	1	2	2	25
18	T S	1	1	2	20	37	D K	1	2	2	25
19	B S	2	1	.	.	38	I K	1	2	3	30

**15.4 Patient Details (continued)**

**A: growth hormone therapy: yes = 1, no = 2; B: common mutation:**

**positive =1 and negative = 2; C: disproportion score (DPS) 1=DPS >1 ;**

**2= DPS <1>-1; 3= DPS <-1; D : growth hormone dose in U/m<sup>2</sup>/week.**

No	Name	A	B	C	D	No	Name	A	B	C	D
39	JM	1	2	2	20	56	IM	2	2	3	.
40	KR	1	2	2	30	57	JG	2	2	2	.
41	MA	1	2	2	40	58	JT	2	2	2	.
42	NG	1	2	2	25	59	KD	2	2	2	.
43	NM	1	2	2	25	60	LJ	2	2	1	.
44	RG	1	2	3	30	61	LM	2	2	3	.
45	RW	1	2	2	30	62	MO	2	2	2	.
46	SW	1	2	2	30	63	PC	2	2	2	.
47	SM	1	2	1	25	64	PW	2	2	2	.
48	WH	1	2	3	30	65	RG	2	2	1	.
49	AM	2	2	3		66	RM	2	2	3	.
50	AN	2	2	3	.	67	SW	2	2	2	.
50	PN	2	2	3	.	68	SS	2	2	3	.
51	DG	2	2	2	.	69	TV	2	2	3	.
52	DD	2	2	1	.	70	TD M	2	2	2	.
53	EW	2	2	2	.	71	TJ	2	2	3	.
54	HP	2	2	2	.	72	TD	2	2	2	.
55	HG	2	2	2	.	73	WD	2	2	3	.

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## **17. DECLARATION**

The work involved in this thesis was performed and completed during my 3 years as a research fellow at The London Centre for Paediatric Endocrinology and Metabolism. Patient recruitments were from endocrinology clinics at The Middlesex Hospital, Mortimer Street, London and Great Ormond Street Childrens Hospital NHS Trust, Great Ormond Street, London.

Auxological measurements were made by accredited measurers in the department during the outpatient visits and I am very grateful for their help in this project. I was responsible for setting up a personal database of all children with Hypochondroplasia and Achondroplasia. Anthropometric data were obtained from growth cards and I was responsible for calculation of standard deviation scores in these patients and subsequent analysis.

Valuable information on early responses to growth hormone therapy was extrapolated from previous studies by Dr. Bridges and Dr. Appan. Some of the older children with achondroplasia and hypochondroplasia had been included in their studies. However, the majority of children within my cohort of cases were those who presented later and had been recruited for growth hormone trials at the London Centre of Paediatric Endocrinology and Metabolism.

A single blood sample was collected from patients and their family members when available. Sample collection was performed by myself in most cases and occasionally by my colleagues within the department of endocrinology. All molecular studies in this project was done by myself under the supervision of Dr. Gill Rumsby, Senior Lecturer, Department of Chemical and Molecular Pathology, Windeyer Building, Cleveland Street, London (University College London Hospitals).

The statistical analysis in this project was performed by myself and supervised by Dr. Helen Spoudeas, Consultant Endocrinologist and Dr. Peter Hindmarsh, Reader in Paediatric Endocrinology

Radiological confirmation of hypochondroplasia was made by consultant radiologists Dr. Chapman, The Middlesex Hospital, London and Dr Hall, Great Ormond Street Childrens Hospital, London.

Ethical approval for this study was obtained from the joint UCL/UCLH committee on the Ethics of Human Research: reference number 95/108. Informed consent was obtained from parents and children (where appropriate). I am grateful for the three years as a research fellow being funded by The National Health Service Executive Responsive Funding Programme, Children Nationwide Medical Research Fund and Pharmacia-Upjohn who supplied the recombinant human growth hormone used in this study.



## **18. PUBLICATIONS ARISING FROM THIS THESIS**

Ramaswami U, Rumsby G, Hindmarsh PC, Brook CG 1998 Genotype and phenotype in hypochondroplasia. *J Pediatr* 133(1): 99-102

Ramaswami U, Hindmarsh PC, Brook CGD 1999 Growth hormone therapy in hypochondroplasia. *Acta Paediatr (Suppl)* 88: 116-117

Ramaswami U, Rumsby G, Spoudeas HA, Hindmarsh PC, Brook CGD 1999 Treatment of Achondroplasia with growth hormone: Six years experience. *Ped Res* 46(4): 435-439

## **PRESENTATIONS AND ABSTRACT PUBLICATIONS**

5th Joint Meeting of the European Society of Paediatric Endocrinology & Lawson Wilkins Pediatric Endocrine Society, Stockholm, 1997.

Ramaswami U, Rumsby G, Brook CGD, Hindmarsh PC. Skeletal and genetic heterogeneity in Hypochondroplasia. *Hormone Research* 1997; 48(2): 69

American Endocrine Society Meeting, New Orleans, 24th - 27th June 1998

Ramaswami U, Rumsby G, Hindmarsh PC, Brook CGD.  
Recombinant human growth hormone therapy in 35 children with Achondroplasia

European Society of Paediatric Endocrinology, Florence, Sept 1998

Ramaswami U, Rumsby G, Spoudeas HA, Hindmarsh PC, Brook CGD. The value of mutation analysis in predicting the anthropometric response to growth hormone therapy. *Horm Res* 1998; 50(suppl): 48