ANALYSIS OF VARIABLE EXPRESSIVITY IN NEUROFIBROMATOSIS 1

by

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B.Sc., McGill University, 1995

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Medical Genetics)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

August 2001

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Date AUG 2/01

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ABSTRACT

Neurofibromatosis 1 (NF1) exhibits extreme clinical variability. This variability greatly increases the burden for affected families. The relationship of genetic factors to variable expressivity in NF1 is poorly understood. To improve understanding of NF1, I studied relationships between several disease features in individuals and among affected relatives. My studies used clinical information on 4731 NF1 patients from three independent databases: the National NF Foundation International Database, the NF Institute Database and a population-based registry of NF1 patients in north-west England.

My initial studies found associations between several *pairs of features in affected probands* and between the occurrence of *individual features in affected parents and children.* This establishes that some patients are more likely than others to develop particular NF1 features. Furthermore, the results of my logistic regressive models are consistent with grouping 9 of the features into three sets of associated features: 1) café-au-lait spots, intertriginous freckling and Lisch nodules; 2) cutaneous, subcutaneous and plexiform neurofibromas; and 3) macrocephaly, optic glioma and other neoplasms. Also, the occurrence of Unidentified Bright Objects on magnetic resonance imaging in young (<21 years) NF1 patients was associated with other expressed diagnostic features. Clinical features within a group may share pathogenic mechanisms that differ, at least in part, from those underlying features in other groups.

I found no local associations between the presence of cutaneous neurofibromas, plexiform neurofibromas, and café-au-lait spots in each of ten divisions of the body surface in NF1 patients. However, the correlation among relatives in the number of body segments affected with one or more lesions was positive and significant for all three

features. The developments of cutaneous neurofibromas, plexiform neurofibromas, and café-au-lait spots in NF1 patients are each spatially independent but influenced by familial factors.

Familial aggregation patterns of NF1 features among various classes of affected relatives were used to examine familial aggregation in greater detail. Using multivariate analyses, statistically significant associations among different classes of relatives were found for several features. Three distinct patterns were observed among the associations for familial features: 1) Lisch nodules and café-au-lait spots had greater associations between 1st degree relatives than between 2nd degree relatives; 2) Subcutaneous neurofibromas, plexiform neurofibromas, café-au-lait spots, and intertriginous freckling had greater associations between sibs than between parents and children; and 3) Head circumference and stature had similar associations for all affected relatives. These familial patterns suggest that unlinked modifying genes, the normal NF1 allele, and the mutant NF1 allele may all be involved in the development of particular clinical features of NF1, but that the relative contributions vary for different features.

The results presented in this thesis suggest that genetic factors *are* involved in phenotypic variability in NF1. These findings also provide specific clues to pathogenesis of NF1 features that can be tested in molecular studies. The methods of biostatistical analysis developed as part of this thesis can be applied to the study of other complex disorders.

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ACKNOWLEDGEMENTS

I would like to thank my research supervisor, Jan Friedman, my friend and co-worker, Patricia Birch, and my statistics guru, Harry Joe, for their guidance and support. I could have never completed this thesis without them. Thank you to my supervisory committee, Barb McGillivray, Diana Juriloff and Dessa Sadovnick, for their encouragement and feedback. To the members of the British Columbia Neurofibromatosis Foundation, thank you for teaching me that medical research can and should be important. Thank you to my family, Carmen Larsen and Ewa Szudek, for their companionship and faith.

1. INTRODUCTION

Neurofibromatosis 1 (NF1) is a generally progressive human disorder that affects the skin, central nervous system, skeleton and other organs. It is inherited in an autosomal dominant fashion and affects around 1/3,500 people (Crowe et al. 1956; Poyhonen et al. 1997b), making it one of the most frequent autosomal dominant diseases in humans. The defining feature of NF1 is the neurofibroma, but patients often also develop other features, such as café-au-lait spots, Lisch nodules, pseudarthrosis, scoliosis, optic glioma and other neoplasms. Some of the features can be serious or life-threatening. Malignancy and vasculopathy are significant sources of morbidity (Zöller et al. 1995; Rasmussen et al. 2001). Owing to the progressive nature of NF1, the ability to diagnose NF1 in a patient improves with age as more features develop or existing features become more numerous or prominent (DeBella et al. 2000b).

1.1 History

Neurofibromatosis was first clinically recognised by von Recklinghausen in 1882 (Crump 1981). Since then, there have been several seminal contributions. The distinction between NF1 and NF2 was made in 1981 (Riccardi 1981). An NIH conference in 1988 (NIH 1988) developed consensus criteria for making the diagnosis of NF1. The NF1 gene was localised to the long arm of chromosome 17 (Goldgar et al. 1989) and cloned in 1991 (Marchuk et al. 1991).

1.2 Prevalence

The prevalence of NF1 has been measured in several different populations. Table 1.2 summarises the methods and results. Prevalence estimates range from 1/960 to 1/7,800, but most are between 1/2,500 and 1/4,500. Ascertainment differences probably contribute to the variation among the studies, but racial differences have not been ruled out. Niimura (1990) surveyed 1,042 NF1 patients in Japan, but did not report prevalence.

1.3 Diagnosis

A person is diagnosed with NF1 if he or she satisfies two or more of the seven criteria listed in the following table (NIH 1988; Gutmann et al. 1997):

Table 1.1: National Institutes of Health diagnostic criteria for neurofibromatosis 1.

Cardinal Clinical Features (Any two or more are required for diagnosis)
• Six or more café au lait spots over 5mm in greatest diameter in prepubertal individuals or over 15 mm in greatest diameter in postpubertal individuals.
• Two or more neurofibromas of any type, or one plexiform neurofibroma
• Freckling in the axillary or inguinal regions
• Optic glioma
• Two or more Lisch nodules (iris hamartomas)
• A distinctive osseous lesion such as sphenoid dysplasia or thinning of the long bone cortex with or without pseudarthrosis.
• A first degree relative (parent, sibling, or offspring) with NF1 by the above criteria

These criteria are very effective for making the diagnosis in adults or older children, but they cannot diagnose 5% of 8-year-olds with NF1 and almost half of 1-year olds (DeBella et al. 2000b). For this reason, other clinical features such as unidentified bright objects on magnetic resonance imaging have been proposed as additional diagnostic

Table 1.2: Studies of the prevalence of neurofibromatosis 1.
Adapted from Rasmussen and Friedman (2000).

Study	Prevalence	Study Site	Population Size	Ascertainment
Crowe et al. 1956	1/2,500-1/3,300	Michigan	252,092	Survey of general hospital admissions and mental institutions
Sergeyev 1975	1/7,800	Russia	94,000	Physical examination as part of evaluation of fitness for military duty. Those with 6 café-au-lait spots examined in detail.
Samuelsson and Axelsson 1981	1/4,600	Sweden	440,082	Medical records, queries of institutions and physicians, assessment of relatives of affected cases
Huson et al. 1989a	1/4,150	Southeast Wales	668,100	Medical records, queries of physicians, assessment of relatives of affected cases
Fuller et al. 1989	1/2,190	New Zealand	113,700	Medical records, queries of physicians, assessment of relatives of affected cases
Clementi et al. 1990	1/4,292	Italy	2,375,304	Medical records from genetics services and hospitals
Garty et al. 1994	1/960	Israel	374,440	Physical examination as part of evaluation of fitness for military duty.
Poyhonen et al. 1997b	1/3,716	Finland	732,000	Medical record review

criteria (Curless et al. 1998), although they must be better characterised before their use as diagnostic criteria is justified (DeBella et al. 2000a).

1.4 Prevalences of clinical features

Ritter and Riccardi studied 111 3-generation families with NF and found no instance of skipped generation, suggesting that penetrance of NF is complete (Ritter and Riccardi 1985). Although penetrance appears to be complete, NF1 can involve many different clinical features. Table 1.3 summarises the prevalences of NF1 clinical features from several clinical studies. Sample sizes are given under the authors' names. The prevalence of every feature was not reported in all of the studies, and a blank cell means that the study did not examine that feature. Nevertheless, each feature was reported in at least two studies, and comparisons can be made.

Riccardi ascertained his subjects through the specialised Baylor NF Program (Riccardi 1992). Friedman and Birch ascertained their subjects through the National Neurofibromatosis Foundation International Database (NFDB), which collects standardised information from specialised clinics around the world (Friedman et al. 1993). Subjects in both studies were drawn mostly from pediatric centres, an ascertainment which contributes to the low mean ages of the samples. The ascertainment of subjects by Crowe et al., Huson at al. and Samuelsson and Axelsson is summarised in Table 1.2. The studies of Huson and Samuelsson are population based and provide useful standards for comparison, despite being smaller than the others. Differences among the studies are partly due to inconsistent definitions of feature "presence" or "absence" –

Friedman and Birch used the NIH criteria (Table 1.1), whereas the other studies largely predate the formal criteria. There is no known overlap between the databases.

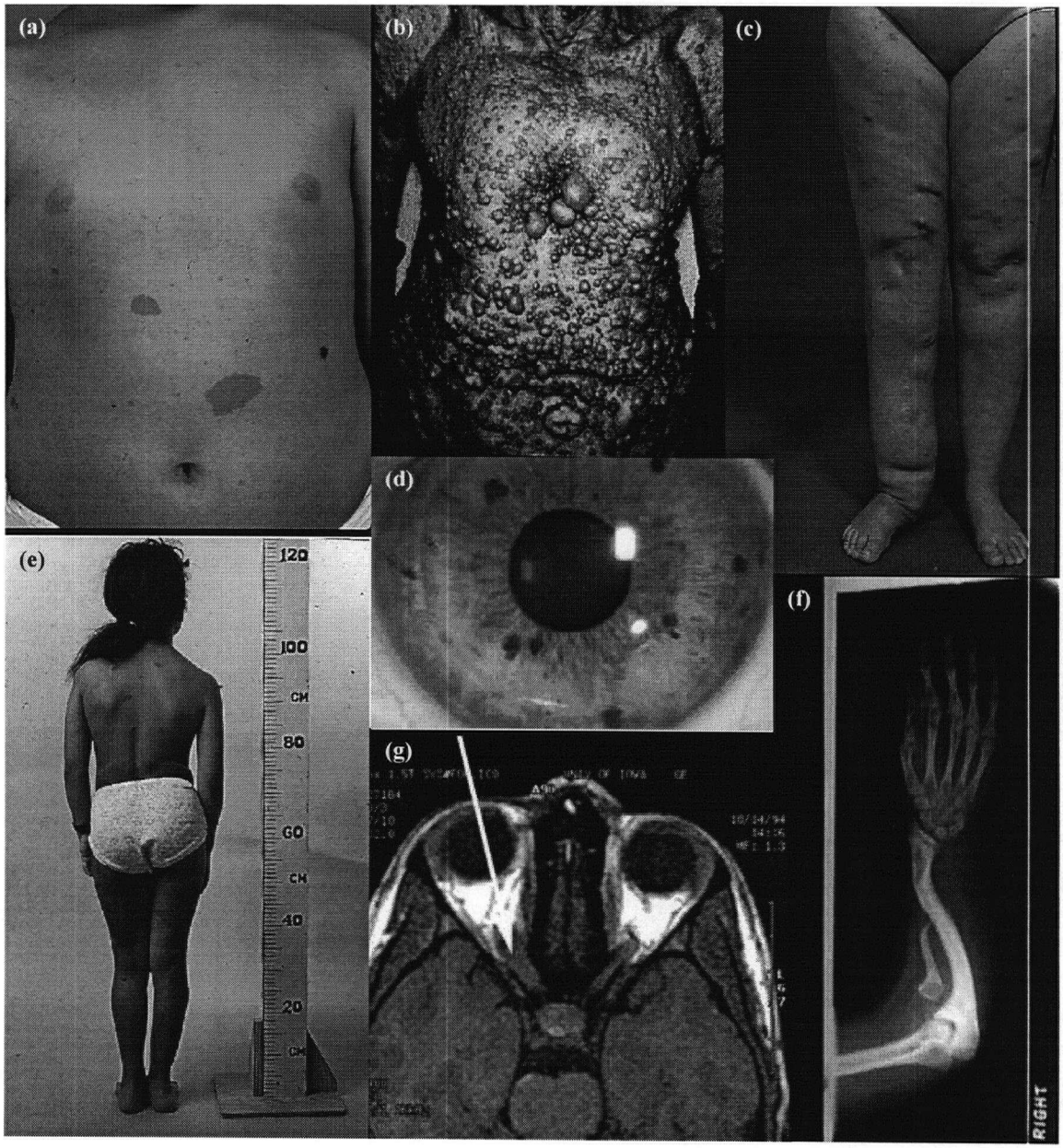
Table 1.3: Comparison of symptom prevalence among 6 studies of NF1 cases.

Feature	Friedman and Birch 1997b (n=1728)	Riccardi 1992 (n=953)	Crowe et al. 1956 (n=203)	Huson et al. 1989a (n=135)	Samuelsson and Axelsson 1981 (n=91)
Café-au-lait spots	89%	100%	78%	84%	82%
Dermal discrete neurofibromas	54%				93%
Plexiform neurofibromas	23%	40%	16%	32%	
Xanthogranuloma	2%	2%		1%	
Lisch nodules	59%	84%		85%	
Pseudarthrosis	2%	3%		4%	
Scoliosis	24%	25%	16%	10%	≥10%
Optic glioma (symptomatic)	4%	10%			0%
Malignancy	5%	4%	5%		10%
Seizures	6%	6%		7%	3-9%
Mean age (years)	17.7	17.8	26.1		

1.5 Pathogenesis of selected clinical features

Café-au-lait spots (Figure 1.1(a)) are present within the first year of life in almost all NF1 patients and tend to increase in number and size during the first decade of life (see Figure 4.1) (Riccardi 1982). This makes café-au-lait spots particularly useful in making the diagnosis of NF1 (DeBella et al. 2000b). They are typically 10 to 30 mm in diameter in adults and can occur anywhere on the skin except the scalp, eyebrows, palms and soles (Riccardi 1992). The basal layer of the epidermis contains melanocytes derived from the neural crest. In a café-au-lait spot, these cells characteristically have giant melanosomes

Figure 1.1: Pictures of selected NF1 clinical features.
(a) Front view of an NF1 patient with 2 large café-au-lait spots. (b) Front view of a patient with hundreds of dermal discrete neurofibromas. (c) Leg hypertrophy resulting from a diffuse plexiform neurofibroma in a patient with NF1. (d) The iris of a patient with NF1, showing multiple Lisch nodules. (e) Rear view of an NF1 patient with scoliosis. (f) Radiograph of the right arm of a patient affected with NF1, showing long bone bowing. (g)Magnetic resonance image of a right optic pathway glioma in a patient affected with NF1. Most of these pictures have been published previously (Friedman et al. 1999).


(a)
(b)
(c)
(d)
(e)
120
100
CM
80
60
CM
40
20
CM
(f)
RIGHT
(g)

– specialised intracellular organelles for melanin synthesis (Bolande 1974; Fitzpatrick 1981). Café-au-lait spots typically cause only cosmetic problems (Gutmann et al. 1997).

Intertriginous (skin fold) freckles in NF1 patients are similar in colour to café-au-lait spots but are usually 1 to 3 mm in diameter. Intertriginous freckles derive from a physiological pathway that has nothing to do with light exposure, but once present they darken with light exposure (Fitzpatrick 1981). Their development may be affected by increased temperature, moisture or salinity found in skin folds (Friedman and Riccardi 1999).

Neurofibromas have the same histology whether they occur as part of NF1 or distinct from NF1. The neurofibroma is a complex benign tumour arising in the fascicles of peripheral nerves – an endoneurium surrounds each nerve fibre and a bundle of nerve fibres is surrounded by a perineurium to form a fascicle. Histologically, a local increase in endoneurial matrix within the fascicle is accompanied by a thickened perineurium, Schwann cells that increase in size and number (Harkin and Reed 1969), and an increased number of mast cells and fibroblasts (Giorno et al. 1989). Neurofibromas can be classified into two categories – dermal discrete (Figure 1.1(b)) and plexiform (Figure 1.1(c)). Dermal discrete neurofibromas include cutaneous and subcutaneous neurofibromas. Cutaneous neurofibromas move with the skin when it is stretched, while subcutaneous neurofibromas lie below the skin and do not move with it (Riccardi 1992). Plexiform neurofibromas include nodular and diffuse plexiforms. Dermal discrete neurofibromas are confined to a single fascicle within a nerve, while diffuse and nodular plexiform neurofibromas involve tortuous changes in the nerve fibres of one ore more fascicles (Burger and Scheithauer 1994). Discrete neurofibromas occur in most NF1

patients and tend to increase in number with age (see Figures 4.3-4.5). Patients who present with plexiform neurofibromas usually do so during childhood. Some diffuse and most deep nodular plexiform neurofibromas are asymptomatic and not apparent on surface exam (Riccardi 1992). Neurofibromas include nerve, Schwann, fibroblast and mast cells. Proportions vary both among and within tumours (Erlandson 1991), but 50-80% of neurofibroma cells react with Schwann cell-specific antibodies (Peltonen et al. 1988). Although *NF1*$^{-/-}$ fibroblasts have a greater proliferative capacity than normal or heterozygous fibroblasts *in vitro* (Atit et al. 1999), loss of heterozygosity at the *NF1* locus in neurofibromas occurs primarily in a subpopulation of Schwann cells, and not in fibroblasts or other cells (Kluwe et al. 1999; Rutkowski et al. 2000; Serra et al. 2000). This suggests that Schwann cells are the pathogenetically culpable cells in neurofibromas.

Lisch nodules (Figure 1.1(d)) are almost exclusively found in NF1 patients (NIH 1988). They are uncommon in young NF1 patients but occur in most patients over 10 years of age (see Figure 4.6). Histologically, Lisch nodules consist of aggregations of oval to round cells that form dome-shaped papules (small, elevated areas) on the anterior layer of the iris (Lukacs et al. 1997). Electron microscope observations reveal that Lisch nodules are hamartomas (self-limiting, benign growths) composed of melanocytic cells, most of which contain immature melanosomes (Perry and Font 1982). Lisch nodules are not associated with visual problems or other ocular manifestations (Friedman and Riccardi 1999). Loss of heterozygosity at the *NF1* locus has not been studied in cells from Lisch nodules.

Scoliosis (Figure 1.1(e)) is frequent in NF1 patients, but the degree of spinal curvature is variable. Dystrophic scoliosis involves changes in rib or vertebral shape, or vertebral spacing or rotation and can result in a severe, angular curve in the spine. Dystrophic scoliosis is considered characteristic of NF1 and is distinct from non-dystrophic scoliosis in NF1 patients, which is similar to idiopathic scoliosis (Winter et al. 1979; Calvert et al. 1989; Cox and Southern 1995) and may merely be coincidental to NF1 (Riccardi 1992). Dystrophic scoliosis in NF1 is thought to be a developmental anomaly with an early onset (Funasaki et al. 1994), but dystrophic features can accumulate over time (Durrani et al. 2000). Its pathogenesis may be congenital, but it is not radiologically evident at birth. Several factors have been implicated in dystrophic scoliosis including: bone deterioration (Wilde et al. 1994), intraspinal or paraspinal tumours (Crawford and Bagamery 1986) and dural ectasia (Stone et al. 1987). One possible mechanism is functional failure of the pedicles, which normally join and transfer compressive forces between the lamina and vertebral bodies (Purkiss et al. 2001).

Tibial or other long bone pseudarthrosis consists of a broken bone that does not heal properly. In fact, the etymology of the word "pseudarthrosis" is the Greek for "false joint". The basis is thought to be a congenital defect in bone formation (Riccardi 1999), and patients with this condition usually present at a very young age with a characteristic bowing (Figure 1.1(f)) of a long bone before fracture occurs (Crawford and Bagamery 1986; Rudicel 1987). Very little is known about the natural history or pathogenesis of pseudarthrosis in NF1, although Stevenson et al. (1999) found a male predominance and no parent of origin effect.

Most optic pathway gliomas (astrocytomas) (Figure 1.1(g)) in NF1 involve the optic chiasm or the optic nerves running between the chiasm and the retina (Listernick and Gutmann 1999). Lesions that involve the optic chiasm can interfere with the hypothalamus and cause precocious puberty (Habiby et al. 1995). Unlike sporadic optic gliomas, those in NF1 rarely involve the optic tracts between the chiasm and the visual cortex, and their growth is usually limited (Listernick et al. 1995). *NF1* expression appears to be tightly regulated in astrocytes and may normally inhibit growth at key points in development. *NF1* expression is nearly undetectable in resting $NF1^{+/+}$astrocytes (Gutmann et al. 1996) but can be increased in response to certain stimuli (Hewett et al. 1995). Furthermore, $Nf1^{+/-}$ mice have increased numbers of cerebral astrocytes and increased astrocyte proliferation compared to wild-type littermates (Nordlund et al. 1995; Gutmann et al. 1999). These activities may involve interacting proteins such as Ras (see Section1.9). One of the functions of neurofibromin is to downregulate p21-*ras* activity, and increased activity of p21-*ras* has been observed in sporadic (non-NF1) astrocytomas (Gutmann et al. 1996).

The majority of tumours in NF1 patients are benign (Riccardi 1992), but patients are at increased risk for malignancy compared to unaffected individuals. Although the lifetime risk for any malignancy is only marginally higher, patients (particularly younger ones) are at much higher risk than usual for connective and other soft tissue and brain malignancies (Rasmussen et al. 2001). The most common malignancies in NF1 are astrocytomas, sarcomas, leukemias, pheochromocytomas and rhabdomyosarcomas (Sørensen et al. 1986; Huson et al. 1988; Riccardi 1992; North 1993). Astrocytomas in NF1 patients are usually optic pathway gliomas (described above) but are found at other

CNS sites in about 1% of patients (Gutmann 1999b). The most common sarcomas are malignant peripheral nerve sheath tumours (MPNST), which usually arise from plexiform neurofibromas (Korf 1999a). Leukemias include juvenile chronic myeloid leukemia (JCML) and myelodysplastic syndromes. Although leukemia is more common in NF1 than in the general population, it is still quite rare, and the excess of leukemia in NF1 is limited to childhood (Gutmann 1999b).

Defects in *NF1* function can lead to greater *ras* activity and increased cellular proliferation. *NF1* codes for a tumour suppressor gene (see discussion below), and the development of tumours in NF1 patients may be the result of somatic mutations in the one remaining normal allele. This certainly seems true of JCML, in which loss of heterozygosity (LOH) has been shown for *NF1* and activated *ras* has been linked to myeloid cell proliferation (Bollag et al. 1996). A similar mechanism may be involved in other NF1 tumours. LOH for *NF1* has also been found in astrocytomas (Gutmann et al. 2000) but not in normal white matter (Lau et al. 2000). LOH for *NF1* has been found in pheochromocytomas (Gutmann et al. 1994), in MPNSTs (Skuse et al. 1989; Skuse et al. 1991), and in some cells in dermal discrete and plexiform neurofibromas (Colman et al. 1995) but it is not clear if it is a cause or a result of tumour growth (Korf 1999a).

The pathogenesis of non-tumour features in NF1, such as short stature and macrocephaly, is even less well understood. Scoliosis and early or delayed puberty occasionally influence stature. However, short stature associated with NF1 usually affects the whole skeleton proportionately, and no specific cause is apparent in most cases (Riccardi 1992; Huson 1994). Disease features such as hydrocephalus and plexiform neurofibromas occasionally affect occipitofrontal circumference (OFC).

However, increased OFC among NF1 patients usually has no obvious cause and appears to result from prenatal or neonatal overgrowth of the brain (Riccardi 1992; Huson 1994).

Seizures occur in less than 10% of NF1 patients and are no different than those in the general population (Gutmann 1999a) – which have a prevalence of less than 1% (Wright et al. 2000). They can occur at any age and may include grand mal tonic-clonic, absence, complex partial or petit mal seizures or hypsarrhythmia. Seizures may be a consequence of haploinsufficiency or abnormal function of the *NF1* gene, but relationship between seizures and *NF1* function has not been investigated, and the pathogenesis of this feature in NF1 is unclear.

1.6 Mortality

Many disease features are more prevalent or prominent among older patients (DeBella et al. 2000b), but few longitudinal studies of NF1 have been done. Zöller et al. conducted a 12-year follow-up study of 70 adult NF1 patients in the city of Goteborg, Sweden (Zöller et al. 1995). Mean life expectancy was 61.6 years, significantly lower than the expectancy of 75 years in the general Swedish population. Twenty-two of the patients died during the study, significantly higher than the 5.1 expected based on the general Swedish population. Malignancy occurred in 12 (55%) of the deaths. 10 of the 12 patients with hypertension died during the period of study. No studies have compared the prevalence of hypertension in NF1 patients and the general population.

A study of death certificates identified 3770 persons with NF1 and found a median age at death of 59 years compared with 74 years in the general population (Rasmussen et al. 2001). In addition, people affected with NF1 were significantly more

likely to have soft tissue or brain malignancy or vascular disease listed on their death certificates. This was especially true among younger subjects. Valvular pulmonic stenosis appears to be more common in NF1, but it is unclear whether other vascular disease is also associated with NF1 (Friedman 1999).

1.7 Gene location and structure

The *NF1* gene has been mapped to 17q11.2; Goldgar et al. (1989) summarised the results of the international consortium for NF1 linkage. The gene was identified in 1990 (Viskochil et al. 1990; Wallace et al. 1990). *NF1* has an open reading frame of 8454 nucleotides (Marchuk et al. 1991) and spans 335 kb of genomic DNA (Li et al. 1995). Sixty exons have been identified, and the genomic sequence is available from GenBank (accession number AC004526). *NF1* has one of the highest mutation rates of any gene in the human genome, estimated between 1×10^{-4} and 3×10^{-5} (Sergeyev 1975; Huson et al. 1989a; Clementi et al. 1990). This is comparable to other genes of similar size, such as Duchenne muscular dystrophy (Vogel and Motulsky 1997), and may be partly due to the large number of nucleotides in these genes. Most reported mutations are unique, but some are observed repeatedly (Korf 1999b; Messiaen et al. 2000).

NF1-homologous loci have been detected by fluorescence in situ hybridisation analysis and genomic sequence homology at 2q21, 2q33, 14q11.2, 15q11.2, 18p11.2, 21q11.2 and 22q11.2 (Regnier et al. 1997). These *NF1*-related loci arose through repeated (partial) gene duplications of the *NF1* locus, rather than through a process involving reverse transcription (Bernards 1998). Most human *NF1*-related loci are

closely associated with centromeres, consistent with emergence by pericentromeric interchromosomal transposition (Regnier et al. 1997).

Characterisation of the *NF1* region itself shows that three other genes are embedded in intron 27b. *EVI2A* encodes a 232 amino acid peptide expressed in the brain and bone marrow which is short-lived and appears to have a transmembrane domain (Cawthon et al. 1991). *EVI2B* encodes a 448 amino acid peptide also expressed in the bone marrow but of unknown function (Cawthon et al. 1990). *OMGP* encodes a 416 amino acid peptide expressed on the surface of oligodendrocytes that appears to have an intracellular adhesion function (Mikol et al. 1990). Large deletions of the *NF1* gene inevitably involve loss of embedded genes, but the consequences of homozygous inactivation of any of these 3 genes have not been demonstrated *in vivo* or *in vitro* (Viskochil 1999).

NF1 is sometimes caused by deletions of the entire *NF1* locus. These large deletions cluster around common breakpoints and can affect expressed regions flanking the *NF1* gene, but it remains to be seen if these influence expression of the disease (Dorschner et al. 2000).

1.8 NF1 gene expression

The *NF1* mRNA transcript is 12-14 kb in size (Buchberg et al. 1990). RT-PCR has detected *NF1* mRNA in almost all tissues, but the highest levels are in neurons, Schwann cells, oligodendrocytes, keratinocytes, astrocytes, adrenal medulla and white blood cells (Daston et al. 1992). The protein product of the *NF1* gene is neurofibromin (SWISS-PROT accession number P21359), a peptide over 220 kD in size (DeClue et al.

1991). Neurofibromin is ubiquitously expressed during embryogenesis in mice, with adult-specific tissue expression levels established one week after birth (Gutmann et al. 1995).

Three major alternatively spliced and differentially expressed isoforms of *NF1* mRNA have been identified in humans. The most common isoform includes an alternatively spliced exon within the GTPase-Activating Protein (GAP) related functional domain (Marchuk et al. 1991) (see section 1.9). The inclusion of this exon results in a decrease of GAP function (Andersen et al. 1993). This isoform is found in many animal species and is associated with differentiated cells (Viskochil 1999). Another isoform involving a different alternatively spliced exon is preferentially expressed in muscle (Gutman et al. 1993). A third isoform is found exclusively in the central nervous system during embryogenesis (Danglot et al. 1995).

In addition to alternative splicing , *NF1* is subject to RNA editing. Nucleotide 2914 can be deaminated from a cytosine to a uracil, leading to a premature end to translation (Skuse et al. 1996). This may be a form of inactivation of the normal *NF1* allele, since varying levels of edited *NF1* mRNA have been found in dermal discrete and plexiform neurofibromas and astrocytomas. A higher range of editing was observed in more malignant tumours compared to benign tumours (Cappione et al. 1997).

1.9 Neurofibromin function

Sequence analysis of *NF1* shows a region of marked homology to the catalytic domain of mammalian GTPase-Activating Protein (GAP) and yeast proteins that can downregulate p21-Ras activity (Xu et al. 1990). Functional studies have shown that both

full-length neurofibromin and only the GAP-related domain (GRD) inactivate p21-Ras by stimulating its intrinsic GTPase activity (Bollag and McCormick 1991). The role of activated Ras is complex and varies among different cell types. Anchored on the cytoplasmic side of the mammalian plasma membrane, activated p21-Ras can stimulate proliferation through the Raf-MAK (mitogen activated kinase) pathway and can also inhibit apoptosis through the phosphoinositol 3' kinase (PI3 kinase) pathway. *NF1* mutations generally result in different tumours than *Ras* mutations (Mulvihill 1994), suggesting that other properties of neurofibromin are also involved in tumourgenesis.

Neurofibromin has other biochemical properties that involve its GRD. Neurofibromin GRD interacts with other Ras-like proteins, such as R-Ras (Rey et al. 1994) and with tubulin (Bollag et al. 1993), which contains a domain similar to Ras (Pai et al. 1990; Nogales et al. 1998). This could represent an additional activity of neurofibromin or a means by which its interaction with Ras is regulated (Viskochil 1999). In addition, neurofibromin GRD activity is differentially inhibited by several different lipids (Golubic et al. 1991) and prostaglandins (Han et al. 1991).

The *NF1* homologue in *Drosophila melanogaster* acts as an activator of the cAMP pathway as well as a negative regulator of Ras (Guo et al. 1997). $NF1^{-/-}$ mutant flies can be rescued not only by an *NF1* transgene but also by expression of activated cAMP-dependent protein kinase A (PKA) (The et al. 1997). This suggests that PKA functions downstream of or parallel to neurofibromin in *Drosophila*. Neurofibromin may also interact with the cAMP pathway in humans. Analysis of *NF1* mutations in NF1 patients suggests a second functional domain upstream of the GRD (Fahsold et al. 2000). This cysteine/serine-rich domain contains PKA binding sites (Marchuk et al. 1991). This

domain is subject to phosphorylation (Izawa et al. 1996) and may influence neurofibromin's interaction with microtubules (Gregory et al. 1993) and cAMP-dependent signalling (Guo et al. 1997; The et al. 1997).

The finding of somatic mutations, or second "hits", in neurofibroma Schwann cells (Kluwe et al. 1999; Rutkowski et al. 2000; Serra et al. 2000), optic gliomas (Gutmann et al. 2000), pheochromocytomas (Xu et al. 1992), JCML (Bollag et al. 1996), and malignant peripheral nerve sheath tumours (Skuse et al. 1989; Skuse et al. 1991) in NF1 patients supports the role of neurofibromin as a tumour suppressor. However, loss of heterozygosity has not been extensively studied in other NF1 features, such as café-au-lait spots, freckles or Lisch nodules. Mutation of both *NF1* alleles may be involved in the development of some NF1 features, but not for others.

It is not known exactly how abnormal or insufficient neurofibromin can trigger the clinical features typically found in NF1 patients, but Ras and cAMP pathways may be involved. Haploinsufficiency of neurofibromin increases growth of mast cells (Ingram et al. 2000), which may be involved in neurofibroma formation (Riccardi 1990). Altered growth has also been demonstrated for keratinocytes resulting in hyperpigmentation and skin cancer among $Nf1^{+/-}$ mice exposed to chemical carcinogens (Atit et al. 2000). A higher ratio of active to inactive Ras has been found in neurofibromas and malignant peripheral nerve sheath tumours (Guha et al. 1996).

Activated PKA is known to stimulate proliferation in some cell types and may normally contribute to body growth (Miyazaki et al. 1992; Kim et al. 1997). This may account for abnormalities in NF1 such as a smaller skeleton in patients with short stature, and abnormal bone formation in pseudarthrosis and scoliosis. Reduced *NF1* expression

results in an increase in glial cell proliferation (Gutmann et al. 1999). Normal stimulation of the PKA pathway accelerates differentiation and inhibits proliferation of glial cells (Raible and McMorris 1990; Raible and McMorris 1993). Deficiencies in this pathway in glial cells may be involved in optic pathway gliomas and brain overgrowth in macrocephaly.

1.10 NF1 gene in other species

The homologue of the *NF1* gene has been cloned and characterised in several organisms. The coding sequence of the *Nf1* locus in mouse has 98% identity to human *NF1* over 2838 amino acids (Buchberg et al. 1990; Bernards et al. 1993; Mantani et al. 1994). The *Drosophila melanogaster NF1* coding sequence has 60% identity to human *NF1* over 2802 amino acids (The et al. 1997). *Caenorhabditis elegans* has 27% identity over 368 amino acids (GenBank Z46266). *Saccharomyces cerevisiae* has 23% identity over 1490 amino acids (GenBank Z36009). The *NF1* gene has been partially characterised in several other organisms as well. Identification of the *NF1* gene in other species has resulted in the development of several animal models.

1.11 Animal models

Animal models of NF1 have added valuable insights into the pathogenesis of NF1 lesions in humans. Although current models have demonstrated only a few of NF1 disease features observed in humans, future models may be even more useful.

Some of these features have been modelled in animals without directly affecting the *Nf1* locus. Transgenic mice expressing the human T-lymphotropic virus type 1 *tax* (HTLV-*tax*) gene develop dermal discrete neurofibromas and occasionally Lisch nodules (Hinrichs et al. 1987; Nerenberg et al. 1987; Green et al. 1992). These neurofibromas appear to contain the same cell types as human lesions and increase in size and number during pregnancy, just as human lesions do. The *tax trans*-regulator represses *Nf1* gene expression through a *cis*-acting element upstream of its transcriptional start site (Feigenbaum et al. 1996). Although repression by *tax* occurs in the absence of mutation at the *Nf1* locus, the mechanism by which it regulates transcription at the *Nf1* locus may shed light on *NF1* expression and disease pathogenesis in humans.

Damselfish exhibit a naturally occurring disorder with tumours resembling human plexiform neurofibromas and regions of hyperpigmentation resembling café-au-lait spots (Schmale et al. 1983; Schmale et al. 1986). The disease is infectious and may be caused by a retrovirus that downregulates function of the damselfish *NF1* gene (Schmale et al. 1996). Viruses have not been implicated in the NF1 gene or disease in humans.

A mouse model has been engineered with a premature truncation in its *Nf1* gene (Jacks et al. 1994). Mice heterozygous for this mutation ($Nf1^{+/-}$)do not develop any of the lesions characteristic of human NF1, while homozygous mutants ($Nf1^{-/-}$) have double outlet right ventricle and die *in utero* (Jacks et al. 1994). Chimeric mice, composed of $Nf1^{+/-}$ and $Nf^{-/-}$ cells, develop multiple plexiform neurofibromas (Cichowski et al. 1999). Compound heterozygotes for both *Nf1* and *Trp53* mutations on the same chromosome ($Nf1^{+/-}$ $Trp53^{+/-}$ cis) frequently develop astrocytomas and malignant peripheral nerve sheath tumours, suggesting that there is biochemical interaction or co-operation between

the products of these two loci (Cichowski et al. 1999; Vogel et al. 1999). Interestingly, the frequency of astrocytomas in these *Nf1*$^{+/-}$ *Trp53*$^{+/-}$ cis compound heterozygotes varies depending on genetic background (Reilly et al. 2000), suggesting that other genetic factors are also involved.

Drosophila melanogaster NF1 mutant heterozygotes are phenotypically normal, but *Drosophila* homozygous for either of two particular *NF1* null mutants have a 20-25% smaller body size (see section 1.9) (The et al. 1997).

A subset of Holstein cattle develops multiple cutaneous neurofibromas, and the trait was found to segregate with a polymorphism near the bovine *NF1* locus (Sartin et al. 1994). However, no specific mutation of the bovine NF1 gene has yet been reported.

1.12 Variable expressivity

One of the most remarkable aspects of NF1 in humans is the variability in its phenotype. This variability affects cardinal clinical features as well as disease complications. Although most people with NF1 are not severely affected, some develop a wide variety of serious features and complications. "NF1 can be manifest in so many different ways and in so many tissues and organs from one family to another, from one person to another within a given family, and from one body part to another and from one time to another in a given person..." (Riccardi 1992). This variability confounds clinical management and limits the predictive ability of genetic counselling for affected families. Determining the causes of the variable expressivity in NF1 is a logical step toward elucidating its pathogenesis and improving treatment.

Present knowledge about the natural history of NF1 comes mostly from hospital records (Crowe et al. 1956), specialised clinics (Riccardi 1992), small population samples (Carey et al. 1979; Samuelsson and Axelsson 1981; Huson et al. 1989a), and regional (McGaughran et al. 1999) and international (DeBella et al. 2000b; Szudek et al. 2000b; Szudek et al. 2000a; Szudek et al. 2000c) databases.

Some features of NF1 can vary over an affected individual's lifetime. Cutaneous neurofibromas usually accumulate with age, while café-au-lait spots develop at an early age and then become lighter in colour in older individuals. Optic gliomas often undergo a short period of rapid growth but can then remain dormant and asymptomatic (Listernick and Gutmann 1999).

The disease phenotype is also affected by systemic developmental changes. Adolescence is a time when neurofibromas often become apparent for the first time and/or those already present grow in size (Riccardi 1992). In a study of 105 women with NF1, sixty-four reported growth of new neurofibromas during pregnancy, and fifty-five noted enlargement of existing neurofibromas (Dugoff and Sujansky 1996).

A few studies looked for but did not find significant associations between common clinical features in individuals. In a longitudinal study, Zöller et al. (1995) found that rapid progression at one point in life does not necessarily predict severity later. Easton et al. (1993) found no associations among clinical features within 175 individual NF1 patients. Specifically, no statistically significant relationship was observed among the following clinical features: number of café-au-lait spots, number of dermal discrete neurofibromas, and presence or absence of plexiform neurofibromas, optic gliomas, scoliosis, seizures and remedial education. The study minimised the confounding effects

of age but examined only a small number of NF1 patients, many of whom were related. Furthermore, the study considered only pair-wise associations between features and not associations among several different features at once.

The disease is also inconsistent among affected relatives – more so than can be accounted for by individual covariates such as age. Carey et al. (1979) examined 104 NF1 patients from 30 families with two or more affected relatives and found that three-quarters of families showed significant differences in severity of NF1 between affected individuals. Riccardi et al. (1979) examined 221 NF1 patients from 127 families and found that affected children of individuals with minimal or mild NF1 had a 25% chance of having a moderate or severe phenotype. A population-based study in south-east Wales examined 135 NF1 patients in 69 families (Huson et al. 1989b) and, although intra-familial variability was not assessed quantitatively, the authors found that the severity of NF1 is "extremely variable" within families. Huson (1994) compiled the clinical features of 19 pairs of monozygotic twins with NF1 from the literature. Twins are exactly the same age, so a high degree of concordance might be expected by chance alone. Nevertheless, a significant portion of the monozygotic pairs were discordant for individual features, particularly for plexiform neurofibromas, optic gliomas, scoliosis, seizures, and learning difficulties.

Most clinical studies of familial associations have based their findings on a relatively subjective assessment without appropriate statistical analysis. The most statistically sophisticated study to date is by Easton et al. (1993), who looked for familial clustering of 8 different NF1 features in 175 affected individuals from 48 families. The results suggest that there may indeed be genetic influences on phenotypic variability in

NF1, but also that intra-familial variability is the rule rather than the exception. Most of the features examined, particularly plexiform neurofibromas, optic gliomas and scoliosis, were more often discordant than concordant between affected relatives.

1.13 Possible sources of variable expressivity

The following are possible sources of phenotypic variability in NF1:

1) *Allelic heterogeneity* is the presence of different mutant alleles at the same locus, each capable of producing an abnormal phenotype. An example is the fibroblast growth factor gene, *FGFR2*. Apert syndrome, characterised by craniosynostosis and syndactyly, results from specific mutations in the *FGFR2* gene (Wilkie et al. 1995). Crouzon syndrome, characterised by craniosynostosis but normal limbs, results from mutations in a different portion of the same gene (Reardon et al. 1994). Jackson-Weiss syndrome, in which craniosynostosis is associated with broad halluces (big toes), tarsal-metatarsal fusions and syndactyly, results from a different mutation in the same exon of *FGFR2* as Crouzon syndrome (Jabs et al. 1994).

Also, mutation of the whole dystrophin gene, or at least one of two specific regions, results in a severe disease – Duchenne muscular dystrophy. If the mutation is limited to a different region of the dystrophin gene, the result is a relatively mild disease – Becker muscular dystrophy (Thompson et al. 1991). Muller et al. (Muller et al. 1997) and Mehler (Mehler 2000) reviewed the molecular genetics of these syndromes.

More than 400 different constitutional *NF1* mutations have been reported (Korf 1999b; Fahsold et al. 2000; Messiaen et al. 2000). A more or less consistent phenotype (facial anomalies, learning disability or mental retardation, and large numbers of dermal

discrete neurofibromas) occurs in association with deletions involving the entire *NF1* gene (Tonsgard et al. 1997; Dorschner et al. 2000), but little other evidence has been found of allele-phenotype correlations in NF1. Similar clinical features have been observed among affected members of a few families with the NF1 variants Watson syndrome (Allanson et al. 1991), familial café-au-lait spots (Abeliovich et al. 1995) or familial spinal neurofibromas (Pulst et al. 1991; Poyhonen et al. 1997a; Ars et al. 1998). These observations are consistent with an allele-phenotype correlation, but no particular kind of *NF1* mutation has been found in families with these or other phenotypic variants.

Likewise, different phenotypes can result from the same mutation. Upadhyaya et al. (Upadhyaya et al. 1996) reported two patients with very different NF1 phenotypes who both have the same mutation in exon 37 of the *NF1* gene. Although allelic heterogeneity may be a factor in the variable expressivity in NF1 (differences in the rest of the *NF1* gene were not ruled out), other factors may also be involved.

2) Polymorphic variations in the normal allele could also modify phenotypic effects of the mutant allele. One example of such an effect is the Rh blood factor. Rh-positive blood specimens usually react with anti-Rh serum. Rh-negative specimens usually do not. In some cases, blood specimens give an intermediate, rather than a strong positive or strong negative response to anti-Rh D serum. In several families, this intermediate response was observed only in members having a particular homologous allele at the Rh complex (Vogel and Motulsky 1997).

Although cystic fibrosis is recessively inherited and NF1 is dominantly inherited, the cystic fibrosis transmembrane conductance regulator (CFTR) gene illustrates how variations in the level of normal transcripts can affect disease severity. A significant

correlation exists between the level of the normal CFTR transcripts and the severity of lung disease. A splicing variant of the CFTR gene results in varying levels of normal transcript among different individuals and between different organs of the same individual (Rave-Harel et al. 1997). Since alternate splicing has also been demonstrated for NF1 mRNA (Metheny et al. 1995), such a variant could contribute to the observed variable expressivity in NF1 patients who carry it as their normal NF1 allele.

3) The action of *modifying genes* has been used to explain variable phenotypes in the haemoglobin system, retinitis pigmentosa, cystic fibrosis, and many other genetic diseases. Sickle-cell anemia, caused by homozygosity for a mutation in the β-globin gene, is less severe when accompanied by hereditary persistence of fetal haemoglobin (HPFH), a group of genetic disorders at other loci that produce deregulation of the γ-globin gene. HPFH increases the amount of fetal haemoglobin (HbF) in affected red cells (Odenheimer et al. 1987; Platt et al. 1991), which reduces the aggregation of α-globin triggered by the imbalance resulting from the β-globin mutation. As a result, patients who are homozygous for the sickle cell mutation but have large amounts of HbF have few or no symptoms of sickle-cell anemia. Similarly, sickle-cell disease is associated with less anemia and improved survival in patients with alpha-thalassemia, usually caused by deletions at the Hb α locus (Weatherall 2001).

Peripherin-2 and rom-1 proteins assemble into oligomers involved in photoreceptor development. Individuals who inherit particular peripherin-2 as well as rom-1 mutations are afflicted with retinitis pigmentosa, whereas individuals who inherit only one defective gene at either locus are normal (Loewen et al. 2001).

As mentioned above, variability of splicing the CFTR gene product has been correlated with the extent of lung disease. Inter-individual and organ differences in CFTR splicing (Rave-Harel et al. 1997) may be modulated by overexpression of cellular alternative splicing factors (Nissim-Rafinia et al. 2000). In another example, calcitonin causes calcium and phosphate absorption from the blood and deposition in bone. Calcitonin mRNA is processed differently in the hypothalamus than in the thyroid, resulting in different amounts of two protein variants in the two tissues (Amara et al. 1982). This process is also influenced by splicing recognition factors (Lou et al. 1998). Differentially spliced forms of the *NF1* transcript have been observed in different tissues and stages of development. Since splicing can be influenced by genetic modifiers (Nissim-Rafinia et al. 2000), an individual's splicing machinery may be sufficiently variable to contribute to the phenotypic variability of NF1 (Metheny et al. 1995).

Another argument for the likely importance of genetic modifiers can be made on the basis of neurofibromin's function. Since neurofibromin downregulates p21ras (Xu et al. 1990), other known regulators of the Ras signal transduction cascade or variants of Ras itself may modify neurofibromin's influence and thus the disease phenotype.

In addition, pseudogenes with partial homology to *NF1* have been found on several chromosomes (see section 1.7). Expression of nitric oxide synthase is suppressed by RNA transcribed from a pseudogene in the snail (Korneev et al. 1999). If a homologous *NF1* gene were found to be expressed, it is possible that its product influences *NF1* expression.

By studying related NF1 patients, Easton et al. (1993) correlated genetic relatedness with the likelihood of concordance of NF1 features such as café-au-lait spots,

neurofibromas, scoliosis and optic gliomas. Phenotypic concordance between relatives increased with increased genetic similarity, supporting a modifying effect. The higher frequency of tumours observed among women with NF1 than among affected men (Samuelsson and Axelsson 1981; Airewele et al. 2001) and the higher frequency of pseudarthrosis observed among males (Stevenson et al. 1999) might also be due to genetic modifiers that are differentially expressed in men and women.

4) *Epigenetic effects* are factors that can affect the phenotype without change in the genotype. They include imprinting, which is the differential expression of genetic material depending on whether the material has been inherited from the mother or the father.

An imprinted gene cluster at 11p15.5 has been implicated in Beckwith-Wiedemann syndrome (BWS), a congenital overgrowth disorder (Reik and Maher 1997). The genetics of BWS are complex, and there are 3 major sub-groups of patients – familial, sporadic and those with cytogenetic abnormalities (Maher and Reik 2000). In each of these subgroups, BWS pathogenesis depends on parent-of-origin effects. Maternal transmission is associated with greater penetrance in familial cases. Some sporadic cases are associated with paternal uniparental disomy. Among those with cytogenetic abnormalities, duplications are derived from the father, whereas inversions and translocations are derived from the mother.

Albright hereditary osteodystrophy (AHO) also can be divided into two subgroups: those with features of AHO alone and those with AHO and associated resistance to multiple hormones (Weinstein and Yu 1999). Patients from both groups have a similar ~50% deficiency of the G_s protein and are often found within the same

family (Levine et al. 1986). This variable expressivity is due to tissue specific-imprinting of the G_s gene (*GNAS1*) (Weinstein and Yu 1999). Maternal transmission of the disease leads to hormone resistance while paternal transmission does not (Davies and Hughes 1993).

In NF1, Crowe et al. (1956) and Miller and Hall (1978) suggested that maternal inheritance of the mutation results in a more severe overall phenotype than paternal inheritance. Miller and Hall studied 62 patients from one centre, and the statistical significance of their observation relies on an excess of very severely affected offspring born to affected mothers. There is no evidence that this group was representative of the NF1 population as a whole. Other investigators, using much larger data sets, did not find a parent of origin effect (Carey et al. 1979; Riccardi and Wald 1987; Huson et al. 1989a).

Shannon et al. (1992) examined parent of origin of the *NF1* mutation in 21 children with NF1 and juvenile chronic myelogenous leukemia (JCML). JCML developed in 12 boys and four girls who inherited NF1 from their mothers, and in five boys who inherited the disease from their fathers. Father-to-daughter transmission was not observed. This finding could be explained by imprinting of *NF1*, but requires further investigation.

5) *Non-genetic factors,* such as environmental exposures and trauma, may also be responsible for some of the variability in NF1. For instance, tissue injury seems to play a role in the development of at least some neurofibromas (Riccardi 1992). Systemic changes during pregnancy (Dugoff and Sujansky 1996) and adolescence (Riccardi 1992) lead to changes in the number and/or size of neurofibromas. Infectious agents can influence NF1 expression in mice and animals (see section 1.11), but this phenomenon

has not been observed in humans. By far the most important non-genetic factor affecting the NF1 phenotype is age. Riccardi (1992) has noted that, for example, the number of peripheral neurofibromas increases with age.

Chance seems to account for some of the phenotypic variability of NF1 (Riccardi 1992; Easton et al. 1993). Evidence of loss of heterozygosity at the NF1 locus in neurofibrosarcomas, peripheral neurofibromas and several other tumours (see sections 1.5 and 1.9) suggests that random "second hit" mutations in somatic tissues may be important in development of the tumours characteristic of NF1. Scoliosis can be caused by plexiform neurofibromas exerting pressure on the spine. The location of a plexiform neurofibroma under the skin is probably affected by chance and, therefore, chance is likely to be involved in the development of scoliosis in many cases (Riccardi 1992).

For haemoglobinopathies, cystic fibrosis and craniofacial syndromes, there is evidence that more than one of the above five possibilities plays a role in the observed variable expressivity (Muller et al. 1997; Mickle and Cutting 2000; Serjeant 2001). These diseases are very different from NF1, but they demonstrate the different genetic mechanisms that can contribute to variable expressivity in humans. The work on NF1 is not as developed as for these diseases, but it is likely that several of the factors from this list and interactions between them contribute to its variability.

1.14 Hypotheses

The hypotheses tested in this thesis are:

1) that subgroups of NF1 patients can be identified who are more likely to develop particular disease features,
2) that some NF1 features cluster in certain families, and
3) that familial aggregation of features, and thus expressivity of NF1, is influenced by genetic factors, such as allelic differences or modifying genes.

1.15 Objectives

My objectives were:

1) to estimate risks of various disease features in clinically-defined subsets of NF1 patients,
2) to test for and estimate associations between the occurrence of features in individual NF1 patients and affected family members, and
3) to examine genetic effects on the occurrence of disease features among relatives with NF1, in order to gain insight into the sources of the variable expressivity.

2. ASSOCIATIONS OF CLINICAL FEATURES IN NEUROFIBROMATOSIS 1

2.1 *Hypotheses*

Clinical features of neurofibromatosis 1 (NF1) do not occur independently in: (1) affected individuals or (2) between affected relatives.

2.2 *Objectives*

To test for and estimate: (1) associations between pairs of clinical features in NF1 probands, and (2) associations of individual features between affected parents and children.

2.3 *Introduction*

Substantial variability is well documented in NF1 among affected individuals and families (see section 1.13). Nevertheless, there is evidence of intra-familial correlations for several NF1 clinical features (Easton et al. 1993) and a few rare NF1 phenotypes appear to "breed true" in families (Allanson et al. 1991; Pulst et al. 1991; Abeliovich et al. 1995; Poyhonen et al. 1997a; Ars et al. 1998).

Recognition of clinical heterogeneity within a disease may provide important pathogenetic insights. For example, understanding that neurofibromatosis 1 and 2 are different diseases was a seminal contribution (Riccardi 1982). In order to determine if clinical heterogeneity exists within NF1 itself, I tested three large clinical data sets for associations between pairs of clinical features in probands. I also tested for familial determinants of clinical variability by looking for associations of individual clinical features between parents and children with NF1.

2.4 Subjects and methods

2.4.1 Patients and data description

All patients included in this analysis were diagnosed with NF1 according to established clinical criteria (Table 1.1) (NIH 1988; Gutmann et al. 1997). The study was performed using clinical data from three independent sets of NF1 patients. At the time of this analysis, the National Neurofibromatosis Foundation International Database (NFDB) (Friedman and Birch 1997b) contained descriptions of 2509 NF1 probands, 211 affected parents and 289 of their affected children. 83% of the NF1 cases are Caucasian, 7% Asian, and 4% African-American. The remaining 6% are mostly combinations of these three ethnic groups. The Neurofibromatosis Institute Database (NFID) (Riccardi 1992) includes standard clinical information on 774 NF1 probands, 132 affected parents and 189 of their affected children. 72% of the cases are Caucasian, 14% Hispanic, 13% African-American and 1% Asian. The Manchester NF1 database (MANF1) is a population-based registry of north-west England and includes clinical information on 270 probands, 94 affected parents and 140 of their affected children (McGaughran et al. 1999). 92% of the cases are Caucasian, 4% East Indian, 2% Black, 1% Bangladeshi and 1% Pakistani. These three databases contain information on many of the same NF1 features. There is no known overlap among the patients included in these three databases. Specific *NF1* mutations have been identified by molecular analysis in fewer than 1% of these patients.

2.4.2 Clinical features

Twelve of the most common or important clinical features of NF1 were selected for inclusion in this study: intertriginous freckling, discrete cutaneous or subcutaneous neurofibromas (referred to as "discrete neurofibromas"), diffuse or nodular plexiform neurofibromas (referred to as "plexiform neurofibromas"), learning disability or mental retardation, Lisch nodules, scoliosis, tibial or other long bone bowing or pseudarthrosis, optic glioma, macrocephaly, short stature, seizures and neoplasms (other than neurofibromas or optic glioma). Café-au-lait spots were not included in this study because they were coded as "present" in all subjects in the NFID, which is incompatible with pair-wise analysis (see below). Table 2.1 summarises the prevalence of these 12 features in the three databases.

Most of the features were identified by physical examination. Discrete neurofibromas were coded as "present" if the subject had two or more cutaneous or subcutaneous neurofibromas. Short stature was coded as "present" if the subject's height was two or more standard deviations below the age- and gender-matched population mean. Subjects with pseudarthrosis, early or delayed puberty, scoliosis, vertebral dysplasia, or spinal compression were excluded from analyses involving height. Macrocephaly was coded as "present" if the subject's head circumference was two or more standard deviations above the age- and sex-matched population mean. Subjects with plexiform neurofibroma of the head, early or delayed puberty, or hydrocephalus were excluded from analyses involving head circumference. Lisch nodules were diagnosed or excluded by a slit lamp examination. The presence or absence of optic glioma was determined by cranial MRI or CT examination; individuals who did not have

cranial imaging were coded as "unknown". Only patients who had definite presence or absence of a feature were considered in comparisons involving that feature.

2.4.3 Statistical analysis in individual probands

Pair-wise combinations of the presence or absence of each feature were analysed in probands by 2-by-2 tables using SAS (1996). The prevalences of many features of NF1 increase with age (Riccardi 1992; Cnossen et al. 1998). Two features that both increase with age may show a strong association because older patients are likely to have both features and younger patients are likely to have neither. Therefore, patients from each database were stratified into 5-year age groups to reduce confounding by age. Patients were also stratified by gender, but not by race because the number of non-Caucasians is heterogeneous and too small for useful comparison. The method of Mantel and Haenszel (1959) was used to estimate a summary odds ratio with 95% confidence intervals over the age and gender strata. This method weighs the proportion in the table for each stratum by its respective sample size, and, therefore, is robust to large differences in sample size among the strata (Everitt 1992). However, it is not likely to be informative in cases where the relationship between the two variables being compared is very different among the strata. Therefore, the Breslow-Day method (SAS 1996) was used to test each triad of odds ratios for homogeneity between the three databases. The test compares the difference between the Mantel and Haenszel summary odds ratio and the odds ratios for the individual strata. Triads with p-values greater than 0.05 were considered homogenous. I performed the analyses in three independent data sets (NFDB, NFID and MANF1) because I expected to observe many associations that

Table 2.1: Prevalence of clinical features of NF1 in probands and affected relatives.
These frequencies vary greatly by age, but all subjects are included in this table to provide an overview of the data sets used in my studies. The presence or absence of most features could not be determined in all patients. The numbers of patients with known feature status are given in parentheses.

	NFDB				NFID				MANF1			
	Probands		Affected Relatives		Probands		Affected Relatives		Probands		Affected Relatives	
Clinical Feature	*%*	*(n)*	*%*	*(n)*	*%*	*(n)*	*%*	*(n)*	*%*	*(n)*	*%*	*(n)*
Freckling	82.9	(2420)	77.2	(452)	75.8	(662)	75.0	(148)	90.8	(228)	81.8	(132)
Discrete NFs	52.5	(2499)	51.0	(467)	51.8	(713)	45.8	(168)	76.0	(242)	54.9	(122)
Plexiform NFs	25.8	(2490)	15.2	(467)	41.5	(743)	25.3	(178)	19.3	(270)	15.2	(171)
Lisch nodules	55.9	(1837)	63.4	(339)	83.0	(395)	89.1	(101)	70.1	(174)	62.1	(66)
Optic glioma	25.0	(1000)	16.8	(125)	21.3	(400)	11.7	(77)	9.5	(190)	12.9	(70)
Seizures	6.8	(2509)	4.3	(470)	5.9	(732)	3.8	(185)	3.0	(237)	9.4	(149)
LD/MR	47.2	(1899)	52.1	(355)	51.1	(587)	48.6	(142)	24.1	(187)	30.0	(100)
Pseudarthrosis	5.2	(2497)	3.9	(462)	4.0	(756)	2.1	(189)	2.1	(243)	2.7	(149)
Scoliosis	25.6	(2498)	14.0	(463)	25.0	(645)	23.1	(156)	14.6	(246)	14.7	(150)
Macrocephaly	20.1	(1553)	17.6	(301)	30.8	(598)	25.5	(137)	24.2	(186)	19.1	(115)
Short stature	12.6	(1903)	20.1	(353)	7.3	(605)	4.0	(124)	34.3	(134)	56.3	(80)
Neoplasms	6.6	(2509)	3.8	(470)	10.7	(774)	9.1	(208)	6.5	(230)	5.8	(137)

NFs: neurofibromas

LD/MR: learning disability or mental retardation

reached nominal statistical significance by chance as a result of multiple comparisons. Odds ratios with 95% confidence intervals that excluded 1.0 in at least two of the three databases were considered unlikely to be due to chance alone. The chance of observing statistical significance for a given pair-wise comparison in two of the three databases and not the third is $3\times0.05\times0.05\times0.95=0.007$. The likelihood of significance in all three databases is relatively negligible. Since 66 different pair-wise comparisons were made, $66\times0.007=0.47$ comparisons are expected to reach statistical significance by chance alone.

2.4.4 Statistical analysis in affected parents and children

The second analysis included affected relatives. A feature that increases with age may show a strong intra-familial association because the ages of sibs within a family are usually similar. Therefore, I limited my analysis to parents and children, who usually differ in age by at least 20 years. For each of the 12 features, a 2-by-2 table was used to compare the frequency of a given feature in children with NF1 of parents with NF1 who had the feature to the frequency in children of parents who lacked the feature. Each individual was counted only once. Twelve contingency tables were generated separately in each database. Odds ratios with 95% confidence intervals were calculated for contingency tables without blank cells. The Breslow-Day method (SAS 1996) was used to test for homogeneity, and the Mantel-Haenszel method (Mantel and Haenszel 1959) was used to estimate summary odds ratios.

2.5 Results

2.5.1 Associations in individuals

Pair-wise associations between each of the 12 clinical features were tested in 2509 NF1 probands from the NFDB, 774 NF1 probands from the NFID and 270 NF1 probands from the MANF1 database (Table 2.2). Most of the associations are moderate in strength – positive associations generally have odds ratios in the range of 2.0-3.0 and negative associations have odds ratios in the range of 0.3-0.5. In the NFDB, an odds ratio of 1.0 was excluded from the 95% confidence limits for 26 of 66 associations tested. There were 23 nominally significant positive associations and 3 nominally significant inverse associations. In the NFID, which contains fewer than 1/3 as many cases as the NFDB, an odds ratio of 1.0 was excluded from the 95% confidence limits for 13 of 66 associations tested. Ten of these nominally significant associations were positive and 3 were negative. In the MANF1, which is about 1/9 as large as the NFDB, an odds ratio of 1.0 was excluded from the 95% confidence limits for 5 of 55 associations tested. All of these were positive. Odds ratios could not be calculated in the remaining 11 associations due to blank cells in the contingency tables. Overall, 6 of 66 tested associations between pairs of features are statistically significant and in the same direction in at least two of the databases (Table 2.2). Four of these 6 associations are statistically homogenous ($p \geq 0.05$) between the three databases. One statistically significant inverse association was observed in at least two independent databases. The associations are shown in Table 2.2 as odds ratios for each database and as summary odds ratios for all three databases together.

2.5.2 Parent-child associations

Table 2.3 summarises the associations for occurrence of the 12 features between: 211 NF1 parents and 289 of their NF1 children from the NFDB; 132 NF1 parents and 189 of their NF1 children from the NFID; and 94 NF1 parents and 140 of their NF1 children from the MANF1. The associations are expressed as odds ratios for each database and as summary odds ratios for all three databases together. Odds ratios could not be calculated for one association in the NFBD, three associations in the NFID and two associations in the MANF1, due to blank cells in contingency tables. A summary odds ratio of 1 was excluded from the 95% confidence limits for Lisch nodules, optic glioma, learning disability or mental retardation, macrocephaly and short stature, but not for intertriginous freckling, discrete neurofibromas, plexiform neurofibromas, seizure, pseudarthrosis, scoliosis or neoplasms. Three of the five statistically significant associations are homogenous between the three databases. No significant negative associations were observed.

2.6 Discussion

2.6.1 Associations in individuals

The large number of cases in these three databases enabled me to find significant associations between several common features of NF1 (Table 2.2). The concordance between the findings in the three independent databases is remarkable. About three ($p=0.05$ multiplied by 66) nominally statistically significant associations were expected by chance in each database, and one would expect chance associations to differ in the

Table 2.2: Associations of NF1 features in probands.
Odds-ratios with 95% confidence limits for associations of features among age- and sex-stratified probands with NF1.

Associated Features		NFDB	NFID	MANF1	Homogeneity (p)	*Summary Odds Ratio*
Freckling	Lisch Nodules	1.8 (1.3-2.4)	1.2 (0.6-2.5)	3.2 (1.2-9.0)	0.85	*1.7 (1.3-2.3)*
Discrete NFs	Plexiform NFs	2.9 (2.3-3.6)	1.9 (1.2-2.9)	2.1 (0.7-6.4)	0.11	*2.6 (2.1-3.1)*
Discrete NFs	Lisch Nodules	1.6 (1.3-2.0)	2.6 (1.3-5.4)	2.1 (0.9-4.7)	0.04	*1.7 (1.3-2.1)*
Discrete NFs	Pseudarthrosis	0.5 (0.3-0.9)	0.3 (0.1-0.9)		0.04	*0.5 (0.4-0.8)*
Plexiform NFs	Scoliosis	1.5 (1.2-1.8)	1.8 (1.2-2.6)	1.6 (0.7-3.6)	0.72	*1.6 (1.3-1.9)*
Seizures	LD/MR	2.2 (1.5-3.1)	3.6 (1.6-7.9)	5.7 (1.3-24.5)	0.18	*2.5 (1.8-3.4)*

Statistically significant associations are shaded.
Odds-ratios with 95% confidence limits could not be determined for comparisons in which Contingency tables contained empty cells. The corresponding cells in Table 2.1 are blank.
NFs: neurofibromas
LD/MR: learning disability or mental retardation

Table 2.3: Associations of features between parents and children.
Odds-ratios with 95% confidence limits for associations of features between parents and children with NF1.

Feature	NFDB	NFID	MANF1	Homogeneity (p)	*Summary Odds Ratio*
Freckling	1.9 (0.8-4.7)	0.8 (0.2-3)	1.7 (0.4-7.4)	0.52	*1.5 (0.8-2.8)*
Discrete NFs	1.8 (0.9-3.9)	3.4 (0.7-16.1)	0.5 (0.1-2)	0.13	*1.6 (0.9-2.9)*
Plexiform NFs	0.8 (0.4-1.9)	0.6 (0.3-1.4)	1.7 (0.6-5.3)	0.35	*0.9 (0.5-1.4)*
Lisch Nodules	8.5 (3.3-22.3)	5.8 (0.3-100)	10.5 (1.8-61.2)	0.94	*8.7 (4.2-18.1)*
Optic Glioma		2.0 (0.3-12.7)	27.3 (1.9-395)	0.17	*3.6 (1.1-12.3)*
Seizures	1.2 (0.1-9.3)	7.1 (0.6-84.6)		0.38	*1.7 (0.4-7.5)*
LD/MR	1.3 (0.8-2.2)	5.8 (2-16.6)	16.8 (1.8-160)	0.004	*2.0 (1.3-3.1)*
Pseudarthrosis	5.1 (0.5-49.3)				*1.8 (0.2-14.3)*
Scoliosis	1.7 (0.7-3.8)	1.6 (0.6-4.1)	1.3 (0.3-6.6)	0.96	*1.6 (0.9-2.8)*
Macrocephaly	8.2 (2.9-22.7)	2.0 (0.8-5.2)	2.6 (0.5-12.4)	0.12	*3.5 (1.9-6.2)*
Short Stature	5.9 (2.3-14.8)		2.3 (0.6-9.2)	0.47	*4.2 (2.0-8.6)*
Neoplasms	5.0 (1.0-26.4)		25.5 (1.3-485)	0.001	*1.3 (0.4-3.9)*

Statistically significant associations are shaded.
Odds-ratios with 95% confidence limits could not be determined for comparisons in which contingency tables contained empty cells. The corresponding cells in Table 2.2 are blank.
NFs: neurofibromas
LD/MR: learning disability or mental retardation

NFDB, NFID and MANF1. The reproducibility of my results suggests that these associations are probably not due to chance alone.

Two of the associations in Table 2.2 were not homogenous among the three databases. The summary odds ratio between discrete neurofibromas and Lisch nodules ranged from 1.6 to 2.6. The summary odds ratio between discrete neurofibromas and pseudarthrosis ranged from 0.3 to 0.5 but could not be calculated in the MANF1 because the contingency table contained an empty cell. Although these ranges are not wide, the summary odds ratios should be viewed with caution.

Seizure disorders can lead to cognitive deficit (Goldstein and Reynolds 1999), but a causal relationship has not been demonstrated in NF1 (Hughes 1994). The positive associations observed may reflect shared pathogenetic mechanisms underlying the associated features. For example, NF1 probands with seizures may be more likely also to have learning disabilities or mental retardation than patients without seizures (Table 2.2) because the effect of the *NF1* mutation on brain development is greater in patients who have seizures.

The association observed between the occurrence of plexiform and discrete neurofibromas (Table 2.2) is consistent with the histopathological similarity between these lesions (Harkin and Reed 1969; Burger and Scheithauer 1994). In addition, both kinds of neurofibromas are associated with acquired somatic loss or mutation of the normal *NF1* allele in at least some cases (Sawada et al. 1996; Serra et al. 1997). NF1 patients who develop plexiform neurofibromas usually do so during childhood (Riccardi 1992). In contrast, discrete neurofibromas are uncommon in young children but are almost universally present among adults with NF1. The association I observed is much

stronger in younger than in older NF1 patients. The odds ratio was 6.9 among patients under five years old, 3.1 among those 5-9, but only 1.3 among those over 40. This raises the interesting possibility that NF1 patients with plexiform neurofibromas develop discrete neurofibromas earlier than patients without plexiform lesions – a hypothesis that can only be tested with longitudinal data.

Previous studies found both discrete neurofibromas and intertriginous freckling to be more common in NF1 patients with Lisch nodules than in NF1 patients without Lisch nodules (Pietruschka 1961; Zehavi et al. 1986), but the responsible mechanism is unknown. Lisch nodules (Perry and Font 1982) and freckles (Fitzpatrick 1981) are derived from cells of melanocytic origin, and all three lesions involve cells derived from the embryonic neural crest (Weston 1981). This is consistent with the suggestion that NF1 is a neurocristopathy (Huson and Hughes 1994) but does not explain why other neural crest-derived tissues, in which the *NF1* gene is expressed, such as the sympathetic ganglia, thyroid C-cells, and parathyroids, are rarely involved in NF1. Moreover, the involvement of neural-crest derived tissues has not been reported for many NF1 features, such as learning disabilities, dysplastic scoliosis, and tibial pseudarthrosis.

Although plexiform neurofibromas growing near the spine can cause abnormal curvature and result in scoliosis, the association I observed between plexiform neurofibromas and scoliosis does not lose significance when patients known to have plexiform neurofibromas of the trunk are excluded. Furthermore, two different forms of scoliosis may occur in NF1 patients – a dystrophic form that occurs within the first decade of life and is often severe and rapidly progressive, and a milder form that occurs later and resembles idiopathic scoliosis (see section 1.5). The association I observed

involves only early onset scoliosis. Many cases of NF1 come to attention because of pseudarthrosis or scoliosis, but it is not clear why these patients are more likely to also have discrete neurofibromas than patients who lack pseudarthrosis or scoliosis. The pathogenetic basis for these associations is obscure.

Most of the associations observed among probands in this study are moderate in strength – positive associations generally have odds ratios in the range of 2.0-3.0 and negative associations have odds ratios in the range of 0.3-0.5 (Table 2.2). Such pair-wise associations are not strong enough to be useful clinically for predictive classification of patients. Furthermore, the diagnosis of NF1 requires the presence of two or more clinical criteria (Table 1.1) and features included in the criteria are not completely independent from one another. This confounding factor will be addressed in analyses of affected non-probands (Chapter 4), who require the presence of only one clinical feature for diagnosis. Nevertheless, my observation of similar associations in three independent databases strongly suggests that common disease features do not occur entirely at random in NF1 and that some patients are more likely than others to develop particular features. This interpretation contrasts with the view that any NF1 patient may develop any manifestation of the disease (Bernhart and Halperin 1990; Riccardi 1992). Most of the associations I observed have never been noted before.

2.6.2 Parent-child associations

My observations in probands suggest that shared pathogenetic mechanisms underlie several common features of NF1. If genetic factors influence these pathogenetic mechanisms, one would expect familial aggregation of such features to occur. Therefore,

I tested for associations between the occurrence of the 12 features among affected relatives.

The ages of sibs within a family are usually similar, and an association may be noted because older sib pairs are more likely to both have a feature and younger sib pairs to both lack a feature that increases in prevalence with age. Parents and children usually differ in age by at least 20 years, so significant associations between the occurrence of a feature in a parent and child are unlikely to be inflated by age confounding. Due to this age difference, I expect the odds-ratios from parent-child comparisons to yield conservative estimates of intra-familial associations. Consequently, I limited my analysis to affected parents and children.

Affected parents with more than one affected child were counted more than once in each 2-by-2 table. This means that the calculated standard errors for odds ratios are underestimates of the true standard errors. The 95% confidence limits in Table 2.3 are slightly narrower than the true 95% confidence limits.

Several strong associations (odds ratios from 2.0 to 8.7) were found by comparing the presence or absence of the 12 features between affected parents and children (Table 2.3). The summary odds ratios for Lisch nodules, optic glioma, macrocephaly and short stature were significant and homogenous among the three databases, although the odds ratio for optic glioma could not be calculated in the NFDB. The NFDB draws its data mainly from pediatric centres and contains no family in which parent and child both have optic glioma. Nevertheless, these associations are probably not due to ascertainment bias. All subjects were assessed in specialised NF clinics, and the family was excluded from a particular analysis if the presence or absence of the feature in question was not

known in both the affected parent and child. The summary odds ratios for learning disability or mental retardation was significant but was not homogenous among the three databases. The summary odds ratio for neoplasms was not significant and not homogenous among the three databases. This may be due to differences among centres in how the feature is diagnosed.

No negative associations were found between affected parents and children. This is consistent with my hypothesis that affected relatives have a more similar NF1 phenotype than unrelated patients. The absence of negative associations also supports the statistical validity of my observations. One would expect to observe negative, as well as positive, associations by chance, if there were really no intrafamilial associations.

I observed familial associations for the occurrence of Lisch nodules, macrocephaly, short stature, and learning disability or mental retardation. In a previous study, Easton et al. (1993) examined 175 individuals with NF1 and found evidence of intra-familial correlations in the number of café-au-lait macules and neurofibromas and in the presence or absence of optic gliomas, scoliosis, seizures and referral for remedial education. Easton et al. observed no correlations for head circumference or plexiform neurofibromas. Furthermore, phenotypic similarity of these NF1 features was found to decrease with decreasing genetic similarity – a trend examined in Chapter 7. Unlike my study, the results of Easton et al. rely heavily on data from six pairs of monozygotic twins. Although the studies differ in design and found different associations both are consistent with the hypothesis that genetic factors influence the phenotypic expression of *NF1* mutations in patients with NF1.

The statistically significant phenotypic similarity among relatives may be evidence of an *NF1* allele-phenotype correlation. Although generally not striking in NF1, phenotypic modification by the nature of the mutant allele has been demonstrated in complete deletions of the *NF1* gene, which tend to result in a severe phenotype (Tonsgard et al. 1997). Other genetic factors that might influence the phenotype in NF1 patients include modifying genes at other loci.

First-degree relatives share half of their DNA sequences at other loci. Similarities at these other loci may contribute to the phenotypic similarities observed in families with NF1. My findings complement those of Easton et al. (1993) and are consistent with their hypothesis that modifying genes influence the NF1 phenotype. The NF1 protein, neurofibromin, is known to interact with many other proteins, including tubulin (Bollag et al. 1993), kinases (Marchuk et al. 1991) and Ras (Buchberg et al. 1990; Xu et al. 1990). Variations in these proteins (Mott et al. 1997; Pepperkok et al. 2000; Saragoni et al. 2000) might also influence the NF1 phenotype .

2.7 Conclusion

Although the NF1 phenotype is highly variable, some patients are more likely than others to develop certain disease features. Genetic factors may influence the particular phenotypic features that develop in many cases. Further clinical, epidemiological, and molecular studies are necessary to elucidate the pathogenesis of this complex disease fully, but my investigations provide hope that some serious complications of NF1 can be predicted or prevented.

3. GROWTH IN NORTH AMERICAN WHITE CHILDREN WITH NEUROFIBROMATOSIS 1

3.1 Hypothesis

Changes in growth affect only a subset of patients with NF1.

3.2 Objective

To analyse the distributions of and generate growth charts for stature and occipitofrontal circumference (OFC) in NF1 patients.

3.3 Introduction

Short stature (≥2 standard deviations below the population mean) and macrocephaly (≥2 standard deviations above the population mean) are more common in people affected with NF1 than in the general population (Weichert et al. 1973; Carey et al. 1979; Huson et al. 1988; Riccardi 1992).

It has been suggested that short stature and macrocephaly are "all-or-none" phenomena that affect only a subset of NF1 patients (Riccardi 1992). According to this hypothesis, NF1 patients would be expected to fall into two distinct groups: (1) those whose stature is in the same normal distribution as unaffected people of the same age and (2) those whose stature is decreased. NF1 patients would also be expected to fall into two distinct groups with respect to macrocephaly: (1) those whose OFC is in the same normal distribution as unaffected people of the same age and (2) those whose OFC is increased. I examined the distributions of these measurements to determine whether changes in growth affect all or only a subset of patients with NF1. I also generated centile curves for stature and OFC by age and gender.

3.4 Subjects and methods

3.4.1 Subjects

All patients included in this study meet the NIH Diagnostic Criteria for NF1 (NIH 1988; Gutmann et al. 1997). Measurements of patient stature and occipitofrontal circumference (OFC) were obtained from the National Neurofibromatosis Foundation International Database (NFDB) (Friedman et al. 1993). At the time of this analysis, the NFDB included extensive demographic and cross-sectional clinical and anthropometric data on 569 Caucasian NF1 patients examined during 1980-98 at 14 participating centres in North America. Information was collected and recorded on each patient using a standard procedure. Patient stature was measured without shoes using a stadiometer. OFC was measured at the largest diameter over the occiput and forehead using a tape measure. The data were subjected to automated range checking and routinely screened for quality and consistency by the database administrator. Only measurements from each patient's first visit to a participating clinic were included in the analysis. Patients who were known to have one or more of the following features on any clinical visit were excluded from analyses of stature: pseudarthrosis (n=22, 3%), early (under 10 years) (n=13, 2%) or delayed (over 15 years) (n=51, 1%) puberty, optic glioma (n=66, 9%), scoliosis (n=98, 14%), vertebral dysplasia (n=19, 3%). The ultimate sample for analyses of stature consisted of 183 males and 202 females. Patients with one or more of the following features were excluded from analyses of OFC: plexiform neurofibroma of the head (n=46, 6%), early or delayed puberty, optic glioma, or hydrocephalus (n=23, 3%). The ultimate sample for analyses of OFC consisted of 216 males and 220 females.

3.4.2 Reference populations

Standard population norms for stature by age were obtained from the National Center for Health Statistics (NCHS) studies during 1963-74 (Hamill et al. 1977). The NCHS standards are based on a sample consisting of 83 percent White or Hispanic subjects and 17 percent Black subjects living in the United States. Standard population norms for OFC by age were obtained from the Fels Institute study conducted during 1929-75 (Hamill et al. 1977). The Fels Institute sample is slightly less heterogeneous than the NCHS sample.

3.4.3 Distribution analysis

Stature and OFC measurements were standardised using z-scores to control for both age and gender:

$$z = \frac{\text{(measurement of patient)} - \text{(mean of the sex- and age- matched control group)}}{\text{standard deviation of the sex- and gender-matched control group}}$$

Patients with stature and OFC measurements corresponding to a z-score with an absolute value greater than 7 were excluded to minimise data entry errors. Four (1%) stature and 2 (0.5%) OFC measurements corresponded to z-scores below -7. One (0.3%) stature and 2 (0.5%) OFC measurements corresponded to z-scores above 7. Single data entry, as used in this study, has an error rate around 2% (Reynolds-Haertle and McBride 1992; Gibson et al. 1994; Horbar and Leahy 1995). After these exclusions, I expect that about 1% of the remaining measurements contain errors.

I tested the standardised data by analysis of variance to determine if significant

differences exist among the measurements made by the major contributing centres.

Distributions of z-scores for stature and OFC were plotted in histograms using SAS (1996). Each histogram is based on the z-scores compiled from males and females of all ages. In addition, the deviation from unimodality of each distribution was quantified by computing its dip statistic (Hartigan 1985). Dip approaches zero for unimodal distributions. The significance of a given dip value is determined by comparing it to the distribution of values from a known unimodal distribution.

3.4.4 Growth curves

Centiles were generated directly from the data for NF1 patients of various ages and compared to the corresponding centiles from reference populations. NF1 patients were divided into gender and age groups matching those of the curves available for population norms. A typical series of age-groups had medians of 2, 2.5, 3...18 years. Age-group limits were determined by splitting the difference between a given median and the next lowest and highest medians. Patients with ages equidistant from two medians were assigned to the older age group. For example, the 2.5 year-old group included patients ages 2.250 to 2.749 years old. The 5th, 25th, 50th, 75th and 95th centiles for stature and OFC were determined for both genders in each age group of NF1 patients and plotted along side the centiles from the corresponding population standards. The data were plotted and smoothed using SAS (1996). Smoothing was done by producing a cubic spline that minimises deviation from fit (Reinsch 1967; SAS 1996). Smoothed curves were inspected to ensure that the final results reasonably represent the data.

Splining was used and described in detail in a study that generated standard curves for the NCHS (Hamill et al. 1977).

3.5 Results

3.5.1 Standardised stature and occipitofrontal circumference

183 males and 202 females were included in analyses of stature. 216 males and 220 females were included in analyses of OFC. Analysis of variance for heterogeneity among the 14 North American contributing centres revealed no centre bias for age- and sex-standardised stature ($p=0.72$) or OFC ($p=0.10$).

Figures 3.1 and 3.2 show the distributions of standardised measurements of stature and OFC among NF1 patients and population norms. Mean standardised stature among NF1 patients is lower than the mean in the reference population. Thirteen percent of the NF1 patients lie two or more standard deviations below the reference population mean, compared to 2% of norms. Mean standardised OFC among NF1 patients is greater than the mean in the reference population. Twenty-four percent of NF1 patients lie 2 or more standard deviations above the reference population mean.

The histograms for stature and OFC appear unimodal (Figures 3.1 and 3.2) – their dip statistics, which measure departures from unimodality, are 0.014 and 0.012, respectively. These correspond to the 10^{th} centiles in distributions of dip based on known unimodal distributions. In other words, the deviations from normality are likely to have resulted from chance alone. The standardised stature distribution has a skewness of 0.32, and a kurtosis of 0.19 – cases are clustered to the left of the mean, and the distribution

Figure 3.1: Distribution of sex- and age-standardised stature.
NF1 patient measurements (histogram) are from the National NF Foundation Database. Unaffected norms (smooth curve) are from the National Center for Health Statistics and the Fels Institute.

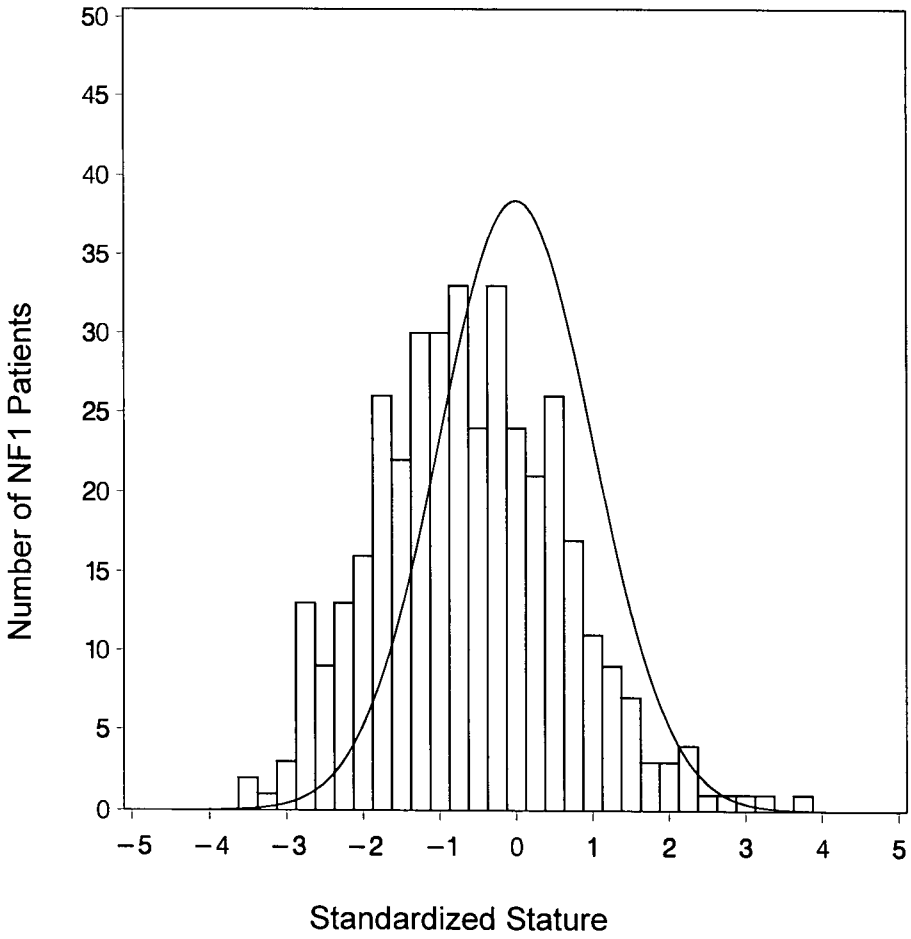
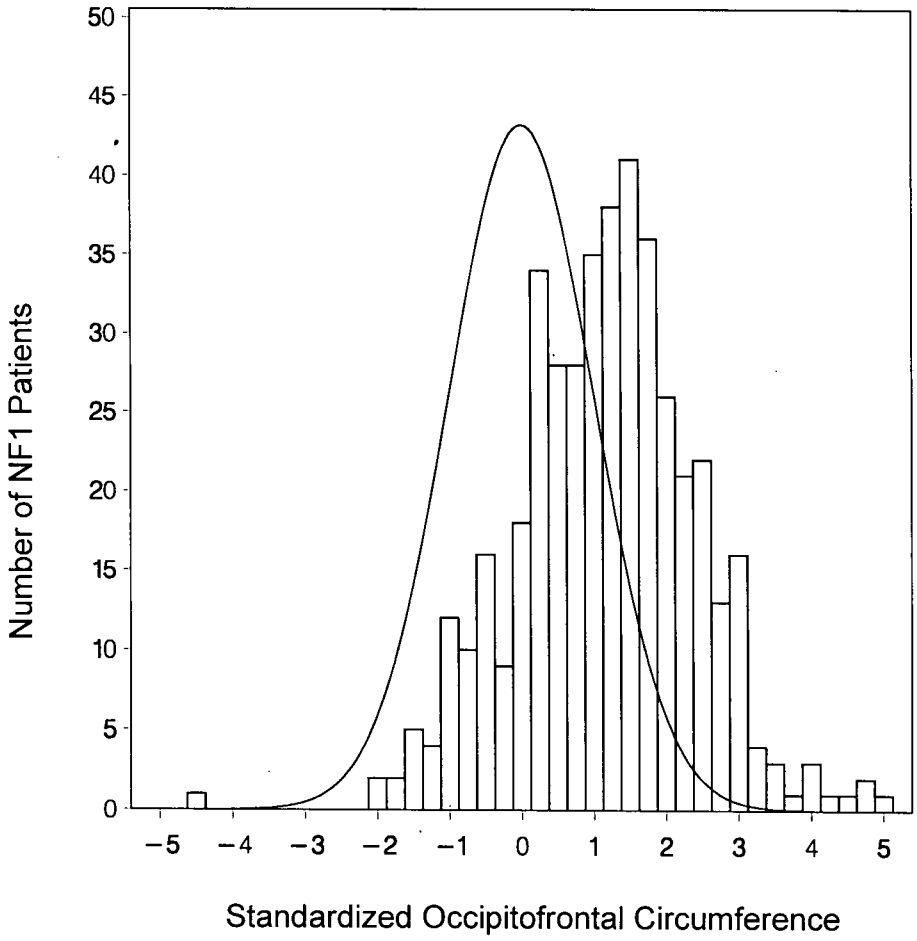

Number of NF1 Patients
50
45
40
35
30
25
20
15
10
5
0
−5
−4
−3
−2
−1
0
1
2
3
4
5
Standardized Stature

Figure 3.2: Distribution of sex- and age-standardised occipitofrontal circumference. NF1 patient measurements (histogram) are from the National NF Foundation Database. Unaffected norms (smooth curve) are from the National Center for Health Statistics and the Fels Institute.


Number of NF1 Patients
50
45
40
35
30
25
20
15
10
5
0
−5
−4
−3
−2
−1
0
1
2
3
4
5
Standardized Occipitofrontal Circumference

peaks more abruptly than a normal distribution. The standardised OFC distribution has a skewness of –0.16 and a kurtosis of 0.87 – cases are clustered to the right of the mean, and the distribution peaks more abruptly than normal.

3.5.2 Centile curves

Stature and OFC centiles by age and gender are shown in Figures 3.3(a)–3.4(b). Median stature is as much as 7 cm lower and OFC 2 cm greater in NFDB NF1 patients than in the standard pediatric growth charts, depending on age and gender.

3.6 Discussion

3.6.1 Population norms

The NCHS and Fels standards were used for comparison to stature and OFC of NF1 patients because these studies cover a wide range of ages and are commonly used clinically to diagnose short stature and macrocephaly. These normal population studies were longitudinal and therefore more accurately represent growth than the cross-sectional studies I used. More recent NCHS standards for boys and girls are available for stature but not for occipitofrontal circumference (http://www.cdc.gov/growthcharts). These new stature standards for boys and girls are remarkably similar to the ones I used (Hamill et al. 1977; Kuczmarski et al. 2000).

Figure 3.3(a): Stature centiles in males 2-18 years.
NF1 patient measurements are from the National NF Foundation Database and are denoted by solid lines. Unaffected norms are from the National Center for Health Statistics and the Fels Institute and are denoted by dashed lines.

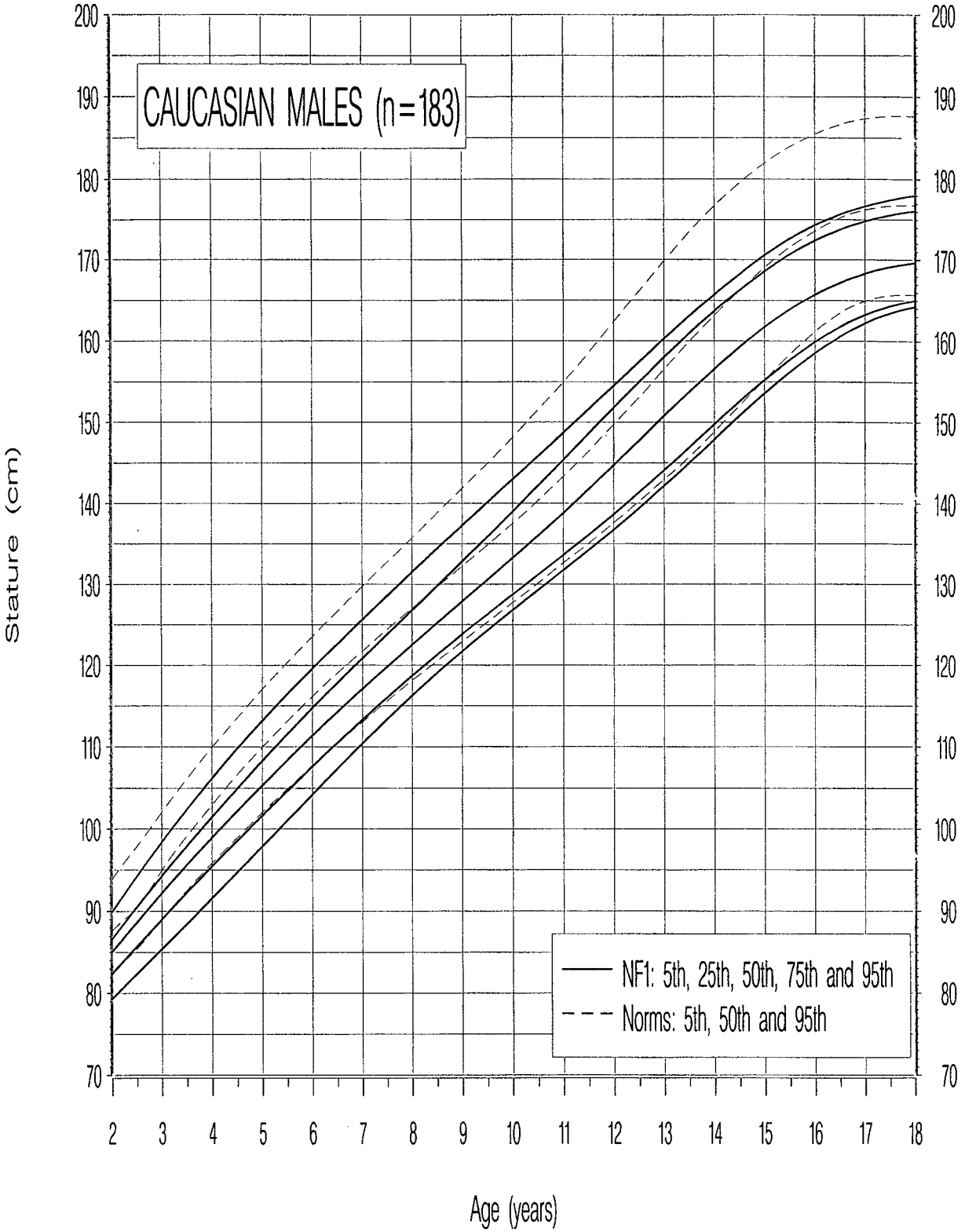

CAUCASIAN MALES (n=183)
Stature (cm)
Age (years)
NF1: 5th, 25th, 50th, 75th and 95th
Norms: 5th, 50th and 95th
200
190
180
170
160
150
140
130
120
110
100
90
80
70
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Figure 3.3(b): Occipitofrontal circumference centiles in males 2-18 years. NF1 patient measurements are from the National NF Foundation Database and are denoted by solid lines. Unaffected norms are from the National Center for Health Statistics and the Fels Institute and are denoted by dashed lines.

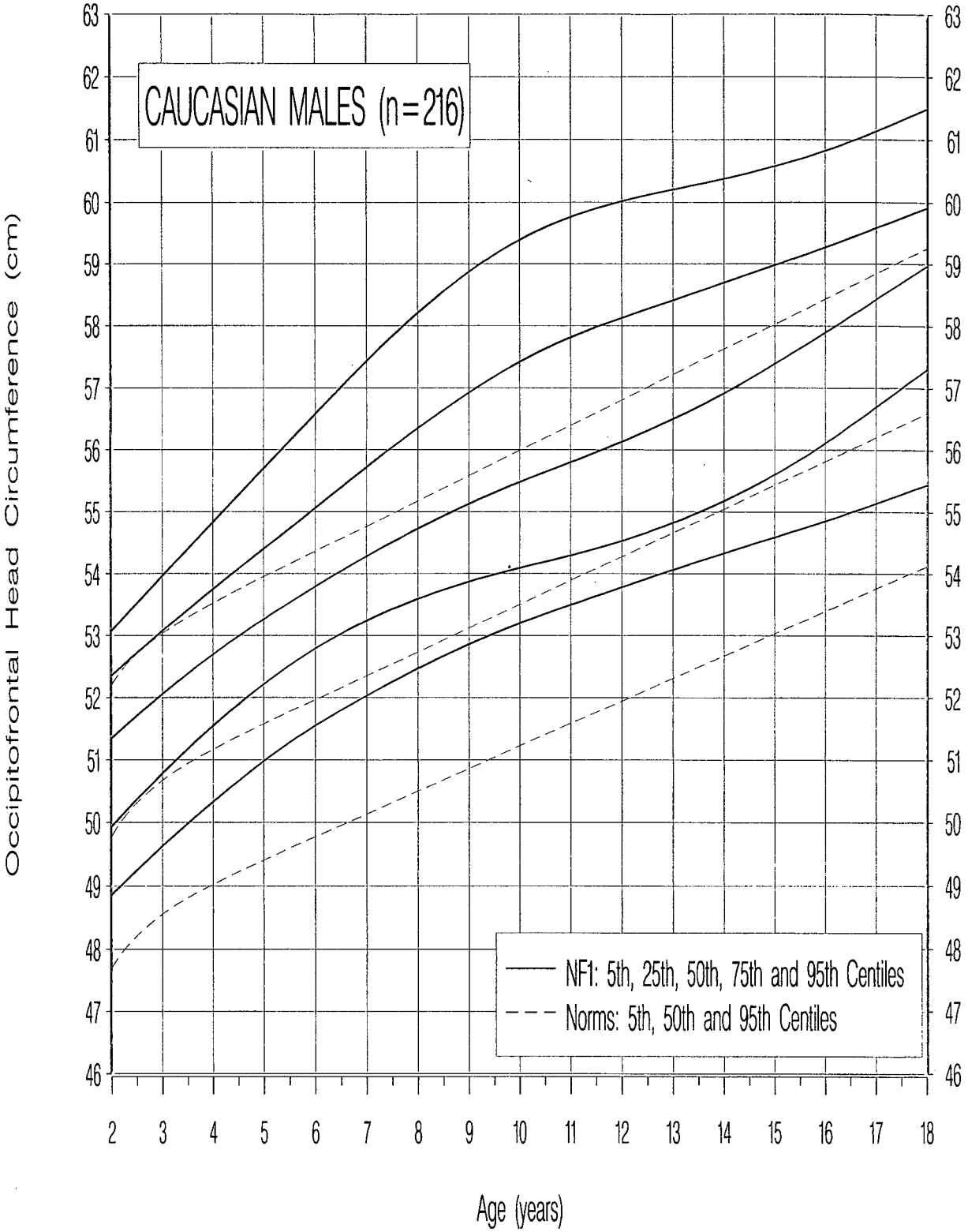

CAUCASIAN MALES (n=216)
Occipitofrontal Head Circumference (cm)
Age (years)
NF1: 5th, 25th, 50th, 75th and 95th Centiles
Norms: 5th, 50th and 95th Centiles
63
62
61
60
59
58
57
56
55
54
53
52
51
50
49
48
47
46
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Figure 3.4(a): Stature centiles in females 2-18 years.
NF1 patient measurements are from the National NF Foundation Database and are denoted by solid lines. Unaffected norms are from the National Center for Health Statistics and the Fels Institute and are denoted by dashed lines.

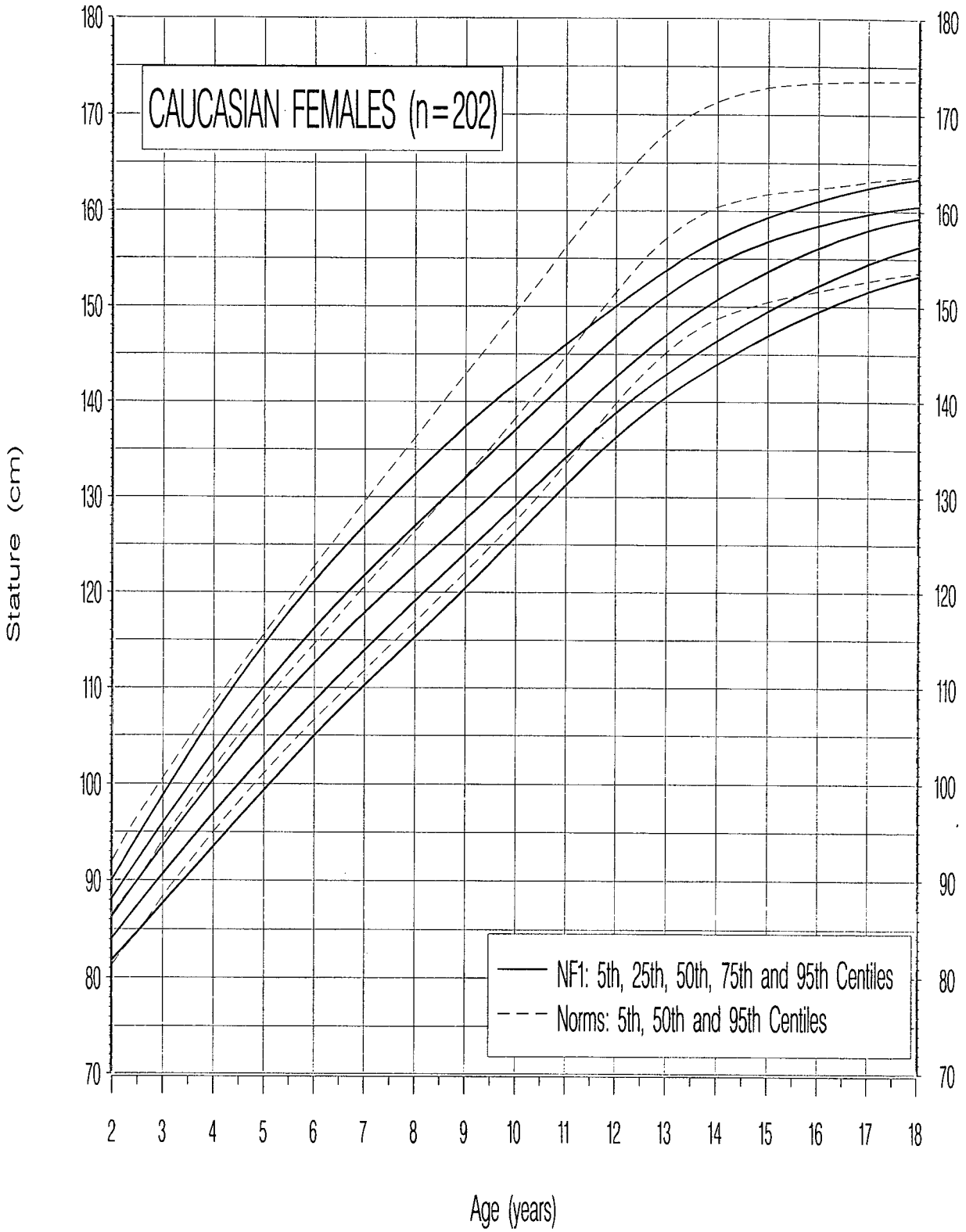
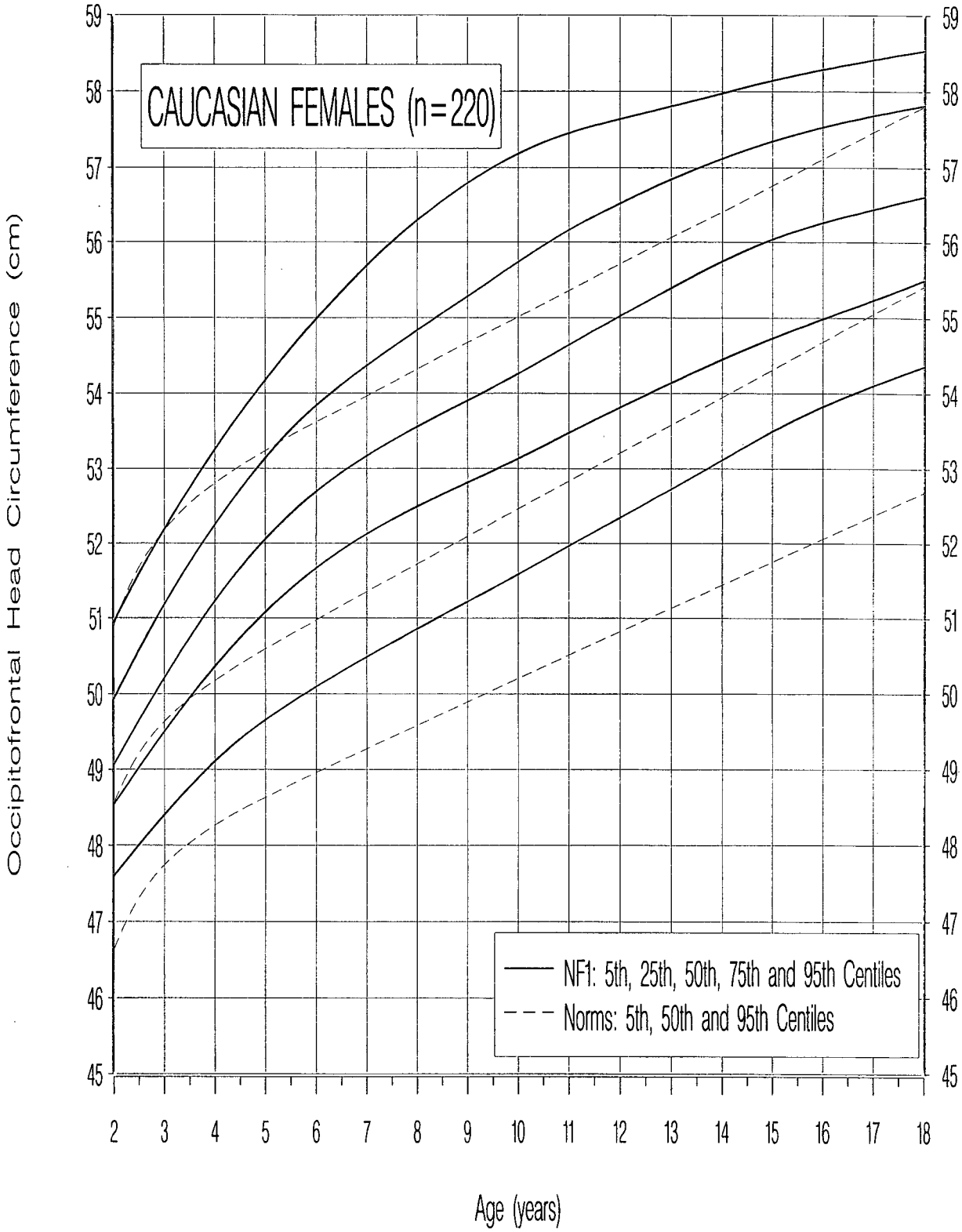

CAUCASIAN FEMALES (n=202)
Stature (cm)
Age (years)
NF1: 5th, 25th, 50th, 75th and 95th Centiles
Norms: 5th, 50th and 95th Centiles
180
170
160
150
140
130
120
110
100
90
80
70
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Figure 3.4(b): Occipitofrontal circumference centiles in females 2-18 years. NF1 patient measurements are from the National NF Foundation Database and are denoted by solid lines. Unaffected norms are from the National Center for Health Statistics and the Fels Institute and are denoted by dashed lines.


CAUCASIAN FEMALES (n=220)
Occipitofrontal Head Circumference (cm)
Age (years)
NF1: 5th, 25th, 50th, 75th and 95th Centiles
Norms: 5th, 50th and 95th Centiles
59
58
57
56
55
54
53
52
51
50
49
48
47
46
45
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

3.6.2 Standardisation

Standardisation for age and gender by z-scores is a transformation that allows pooling of measurements across groups that differ in age and gender. This transformation can be applied to measurements for which standard population distributions are approximately normal. The distributions of stature and OFC satisfy this criterion (Hamill et al. 1977). Thus, a subject's z-score closely corresponds to his or her centile rank.

3.6.3 Assessment of heterogeneity

I was concerned that differences among examiners and equipment at different centres would increase the variability of my sample and diminish my ability to analyse the standardised distributions. However, analysis of variance detected no statistically significant differences for stature or OFC among the contributing centres.

3.6.4 Assessment of standardised distributions

The shifts in the standardised distributions of stature and OFC (Figures 3.1 and 3.2) confirm that, on average, the NF1 patients in this study are shorter and have larger heads than standard populations. The shifts are very similar to those in a recent longitudinal study by Carmi et al. (1999). The population norms were not taken during the same years as my sample, and some were taken 20 or more years earlier. Secular trends suggest that stature and OFC may have increased in the normal population over

this time (Roche 1979; Byard and Roche 1982; Ounsted et al. 1985; SAS 1996). If year-specific standards were used in my study and the one by Carmi et al., the shift in stature may be slightly larger than indicated here and the shift in OFC may be slightly smaller. Ascertainment bias may also affect the distribution shifts. Although short stature and macrocephaly are not among the diagnostic criteria for NF1, these features may have contributed to the patients' referral to the contributing NF clinics (NIH 1988; Gutmann et al. 1997). Therefore, the group in this study may be shorter and have bigger heads than a population-based sample of children with NF1.

The distributions of standardised stature and OFC have positive kurtosis values – they have fewer data points within one standard deviation of the mean than does a normal distribution. This variability could result from several factors: 1) The NFDB patient measurements are cross-sectional and, therefore, more variable than longitudinal data; 2) The NFDB patient group is geographically heterogeneous; 3) A small proportion of cases may have data-entry errors; 4) Ascertainment bias may increase the frequency of outliers; 5) Such distributions might represent composites of more than one normally distributed group with the same mean but different variances (Zar 1999); 6) The estimates of the standard deviation used in the standardisation may have been different than the standard deviation among patients.

Riccardi has suggested that short stature and macrocephaly in NF1 are "all-or-none" phenomena, i.e., that two different groups of NF1 patients exist: those with short stature (or macrocephaly) and those without (Riccardi 1992). Under this hypothesis, the distributions should be bimodal. My findings are not consistent with this suggestion.

The distributions (Figures 3.1 and 3.2) indicate that stature is reduced to some degree and OFC enlarged to some extent in all NF1 patients.

3.6.5 Assessment of centile curves

The centile curves for stature and OFC (Figures 3.3(a)-3.4(b)) are comparable to those from a recent study of Italian NF1 patients (Clementi et al. 1999). Minor differences may be partly due to line-smoothing techniques and geographic variation. Deviation from these NF1 standards may indicate the effect of a specific disease feature such as optic glioma or hydrocephalus. On the other hand, NF1-specific charts may provide reassurance that an affected child's growth, although outside the "normal" range on standard pediatric growth charts, is actually normal for a child with NF1. I also created charts for body mass index and the ratio OFC/stature by age and gender in Caucasian NF1 patients. These charts are available from http://www.medgen.ubc.ca/friedmanlab.

3.6.6 Pathogenesis

Patients with known hydrocephalus and plexiform neurofibromas of the head were excluded from my analyses of OFC, so enlargement of the head in the remaining patients must be due to enlargement of the scalp, skull or brain. In NF1, enlargement of the brain is the likely cause (Riccardi 1992; Huson 1994). Glial cell proliferation is increased *in vitro* by sera from NF1 patients, compared to sera from unaffected individuals (Caronti et al. 1998). Optic or other CNS gliomas are another manifestation

of glial cell proliferation. They were observed by MRI in 10% of NF1 patients in the NFDB. Other studies have observed optic gliomas in 1.5% of 135 and 15% of 217 NF1 patients (Lewis et al. 1984; Huson et al. 1988). Glial overgrowth is an important part of NF1 and it may be responsible for macrocephaly in NF1 patients.

Patients with puberty disturbance or bone abnormalities were excluded from my analyses of stature. The cause of the stature reduction in the remaining NF1 patients is unknown, but is thought to affect the skeleton proportionately (Riccardi 1992). Data reviewed by Howell et al. (1998) indicate that growth hormone replacement therapy resulted in a moderate increase in stature for NF1 patients with biochemical evidence of growth hormone deficiency. Although growth hormone levels were not measured routinely in the NFDB patients, less than 1% are known to have ever had documented growth hormone deficiency. Growth hormone deficiency was found in only 3 (2.5%) of 122 children with NF1 in another study (Cnossen et al. 1997). Stature appears to be reduced in many more NF1 patients than can be attributed to such deficiency.

The findings in this study are consistent with known molecular function of the *NF1* gene and protein in humans and *Drosophila* (see section 1.9). Mutations in neurofibromin in GAP-related domain produce hyperactivity of p21ras which may contribute to increased glial (astrocyte) cell proliferation and to enlargement of the brain in NF1 patients (Nordlund et al. 1993; Rizvi et al. 1999). The *NF1* homologue in *Drosophila* also acts as an activator of the cAMP pathway (Guo et al. 1997; The et al. 1997). *Drosophila* homozygous for either of two particular *NF1* mutants that lack expression of NF1 protein are 20 to 25% smaller than flies of the parental strain (The et al. 1997) – a phenotype rescued by activating the cAMP pathway through expression of

activated protein kinase A. Human neurofibromin also has cAMP dependent protein kinase A (PKA) binding sites (Marchuk et al. 1991; Fahsold et al. 2000). Deficiencies in this pathway may contribute to a smaller phenotype in humans as well. Activated PKA is known to stimulate proliferation in some cell types and may normally contribute to body growth (Miyazaki et al. 1992; Kim et al. 1997). Normal stimulation of the PKA pathway also accelerates differentiation and inhibits proliferation of glial (oligodendrocyte) cells (Raible and McMorris 1990; Raible and McMorris 1993). Neurofibromin involvement in or between the PKA and p21ras pathways may contribute to the larger heads observed in people diagnosed with NF1 (Izawa et al. 1996). However, patients with the smallest stature did not also have the largest heads.

3.7 Conclusion

Short stature and macrocephaly are well-recognised clinical features of NF1. This study suggests that these changes in growth affect all NF1 patients and are not limited to particular subgroups. Therefore, the growth curves presented here can be used for all children with NF1. The mechanisms by which mutations of the *NF1* gene produce these phenotypic effects are unknown, but understanding how they do so may provide an important clue to the pathogenesis of other NF1 features.

4. LOGISTIC REGRESSIVE MODELS OF CLINICAL FEATURES IN NEUROFIBROMATOSIS 1

4.1 Hypothesis

Clinical features of neurofibromatosis 1 (NF1) are not randomly distributed among affected individuals.

4.2 Objective

To identify and estimate moderate to strong associations in individual NF1 probands between several different clinical features and to validate these associations in affected relatives and in a population based sample.

4.3 Introduction

NF1 expressivity is tremendously variable, but subtle phenotypic patterns may exist within subgroups of affected patients. The existence of such subgroups is supported by the observation of a relatively consistent phenotype among patients with deletions of the entire *NF1* and in families with rare NF1 variants (see section 1.13).

In Chapter 2, I demonstrated several associations between pair-wise combinations of clinical features among age-stratified probands with NF1. These analyses support the existence of phenotypic subgroups but were limited in two ways: only two features could be examined at once and some of the comparisons may have been confounded by age. Although I analysed age in 5-year strata, virtually every feature of NF1 exhibits a different relationship with age, and there still may have been considerable age-related variability, especially among the youngest patients.

In another study, numerical taxonomy was used on NFDB data to identify five

distinct clinical groups of NF1 patients (Friedman et al. 1995). However, these clusters were also subject to confounding by age and other variables.

Log-linear models have been used previously to test for associations among several different congenital malformations and determine which features tend to occur together (Beaty et al. 1991). However, log-linear models, like my previous study based on contingency tables, can only treat age as a categorical variable. Since virtually every feature of NF1 exhibits a different relationship with age, I chose to control age as precisely as possible – as a continuous variable.

Unlike log-linear models, logistic regressive models allow the analyst to use continuous variables as well as binary and categorical ones. In this study, I have extended my analysis of associations among clinical features in NF1 patients by using logistic regression to consider joint and interactive effects of several clinical features at once and to control for age as a continuous variable. My findings clarify and refine the associations among clinical features in NF1 patients and provide further clues to the pathogenesis of these features.

4.4 Subjects and methods

This study involved analysis of four separate clinical samples of patients with NF1 – developmental, validation, and relative samples from the National Neurofibromatosis Foundation International Database (NFDB) and a population-based sample from the Manchester NF1 database (MANF1), as described below. Logistic regressive models were built from an initial series of one-covariate models, by progressively adding covariates and interaction terms, in the developmental sample. The

best fitting of these models were then tested in each of the other samples, using both the parameters from the developmental sample and by refitting the parameters in each of the other samples.

4.4.1 Subjects

Subjects were obtained from two large clinical databases: the NFDB and the MANF1. All patients included in this analysis were diagnosed with NF1 according to established clinical criteria (NIH 1988; Gutmann et al. 1997). At the time of this analysis, the NFDB included extensive demographic and cross-sectional clinical information on 2797 NF1 probands and 511 of their affected relatives examined since 1980 at 25 participating centres in North America, Europe and Australia. 83% of the cases are Caucasian, 7% Asian, 4% African-American, 6% other or mixed race. All information was collected and recorded on each patient using a standard procedure (Friedman and Birch 1997b). The data were audited for quality and consistency by the NFDB administrator. The Manchester NF1 Database (MANF1) sample is described in section 2.4.1. There is no overlap among the patients included in the NFDB and MANF1 databases.

4.4.2 Clinical features

I selected 13 important or frequent clinical features of NF1 for this study: café-au-lait spots, intertriginous freckling, discrete cutaneous neurofibromas, discrete subcutaneous neurofibromas, diffuse or nodular plexiform neurofibromas (referred to as

"plexiform neurofibromas"), Lisch nodules, scoliosis, tibial or other long bone bowing or pseudarthrosis ("pseudarthrosis"), optic glioma, macrocephaly, short stature, seizures and neoplasms (other than neurofibromas or optic glioma). Each of these features was coded as either "present", "absent" or "unknown". Age, coded to the nearest 0.01 year, and gender were considered as covariates.

Café-au-lait spots were considered "present" in patients with 6 or more spots of sufficient size (see Table 1.1). Most of the other features were identified according to the criteria described in section 2.4.2. Patients coded as "unknown" for a particular feature were not considered in models involving that feature.

4.4.3 Statistical models

Thirteen separate logistic regression models were built, with the logit of each of the 13 NF1 features analysed set as the binary response variable (Y) in a different model. Whereas linear regression attempts to compute the mathematical relationship between the covariates and the response variable directly, logistic regression attempts to compute the relationship between the covariates and the log of the odds of the response variable being "present". The frequencies of many features change with age, but this effect is not uniform among the features (Friedman et al. 1999). Therefore, age was controlled as precisely as possible, as a continuous explanatory variable. First, a one-covariate model was constructed using age as the only covariate:

$$Y = \log\left(\frac{p(1|x)}{1-p(1|x)}\right) = \alpha + \beta_1 AGE$$

where: $p(1|x)$ is the probability that the feature is "present", given the covariates, x; the logit is the log function on the left side of the equation involving $p(1|x)$; α is the y-intercept; β is the slope; and AGE is the age of the subject at the time the feature was assessed.

Maximum likelihood techniques were used to generate parameter estimates (SAS 1996). Linearity in the logit was examined in each model, and age was transformed when necessary to meet the requirement of linearity in the logit.

$$\log\left(\frac{p(1|x)}{1-p(1|x)}\right) = \alpha + \beta_1 AGETRF$$

where

$$AGETRF = \exp(-c \times AGE)$$

At AGE zero, the value of this function is $\alpha + \beta_1$. For negative values of β_1, the value of the function approaches α as AGE gets larger. This function was used to approximate the frequency-by-age curves of the NF1 features considered in this study. It was necessary to use this transformation of AGE to maintain linearity of the logit for most outcome variables in this study.

A series of two-covariate analyses was then performed using the equation,

$$\log\left(\frac{p(1|x)}{1-p(1|x)}\right) = \alpha + \beta_1 AGETRF + \beta_2 x$$

in which each of the 13 features was set in turn as the response variable, and *AGETRF* and one of the 12 remaining features (x) were used as explanatory variables to screen for potential main effects. Variables with parameters (β's) with $p<0.2$, a standard cut-off value that allows more variables to influence the outcome (Hosmer and Lemeshow 1989), were included as explanatory variables (x_i's) in multi-covariate analyses. *AGETRF* and gender were included as covariates in all models.

$$\log\left(\frac{p(1|x)}{1-p(1|x)}\right) = \alpha + \beta_1 AGETRF + \beta_2 MALE + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_5 \ldots$$

Following maximum likelihood estimation of the parameters in the multivariable model, the importance of each explanatory variable was reassessed. Explanatory variables with parameters greater than zero with $p<0.2$ were used to refit the model and interaction terms (δ's) among the explanatory variables were considered by forward selection. For example,

$$\log\left(\frac{p(1|x)}{1-p(1|x)}\right) = \alpha + \beta_1 AGETRF + \beta_2 MALE + \beta_3 x_3 + \beta_4 x_4 + \delta_1 x_3 x_4$$

4.4.4 Model validation

Fitted logistic regressive models always perform favourably on the sample used to generate them (Hosmer and Lemeshow 1989). Therefore, a random subsample consisting of 1,384 of the 2,797 NF1 probands from the NFDB was excluded, and models were developed on data from the remaining 1,413 NFDB probands (the "developmental sample"). These models were tested on data from the 1,384 NFDB probands who were originally excluded, the "validation sample". The models were also tested on data from 511 affected relatives of the 2797 NFDB probands and on population-based data from the MANF1, which includes both probands and affected family members. The Hosmer and Lemeshow (1989) goodness-of-fit test was used to assess how well the parameter estimates from the developmental sample fit the validation, affected relative, and MANF1 samples. This statistic compares the likelihood of a feature predicted by model parameters to its actual occurrence in the data. Models with a goodness-of-fit $p \geq 0.05$ were considered adequate. In addition, parameters for covariates and significant explanatory variables from the best-fitting models derived in the developmental sample were re-estimated by maximum likelihood in the validation, and affected relative samples, and in the independent MANF1 sample, to allow more detailed comparison.

4.4.5 Interpretation

Logistic regressive models have a straightforward interpretation in terms of odds-ratios. The strength of association between the response variable (Y) and an explanatory variable (x_1) in a one-covariate model is measured by β_1. Subjects with variable x_1 coded as "present" are $\exp(\beta_1)$ times more likely to also have feature Y than are subjects with

feature x_1 absent. The strength of interaction between Y and explanatory variables (x_1 and x_2) in a two-covariate model is measured by β_1, β_2, and δ_1. Subjects with variables x_1 and x_2 present are $\exp(\beta_1+\beta_2+\delta_1)$ times more likely to also have the response feature. Subjects with variable x_1 present and x_2 absent are $\exp(\beta_1)$ times more likely to also have the response feature. Confidence intervals were calculated as $\pm 1.96 \times SE$, where SE is the standard error for β. For odds ratios involving more than one β, confidence intervals were calculated using individual standard errors and their covariances: $\pm 1.96 \times \text{sqrt}(SE_1^2+SE_2^2-2 \times \text{covariance}_{12})$. Odds ratios with 95% confidence intervals that excluded 1.0 were considered unlikely to be due to chance alone.

4.5 Results

4.5.1 Prevalence by age of NF1 clinical features

Whereas Table 1.3 shows feature prevalences for subjects of all ages, Figures 4.1 – 4.13 show the prevalence by age of café-au-lait spots, intertriginous freckling, subcutaneous neurofibromas, cutaneous neurofibromas, plexiform neurofibromas, Lisch nodules, optic glioma, seizures, pseudarthrosis, scoliosis, macrocephaly, short stature and neoplasms (other than optic glioma and neurofibromas). These curves were generated from cross-sectional clinical information on 3308 NF1 patients recorded in the NFDB. The total number of patients included for each clinical feature varies because I excluded cases in which the presence or absence of the particular feature could not be determined unequivocally from available data.

Figure 4.1: Prevalence by age of 6 or more café-au-lait spots.
The curve is based on 3244 NF1 patients from the National Neurofibromatosis Foundation Database. "Presence" required 6 or more café-au-lait spots over 5mm in greatest diameter in prepubertal individuals or over 15 mm in greatest diameter in postpubertal individuals

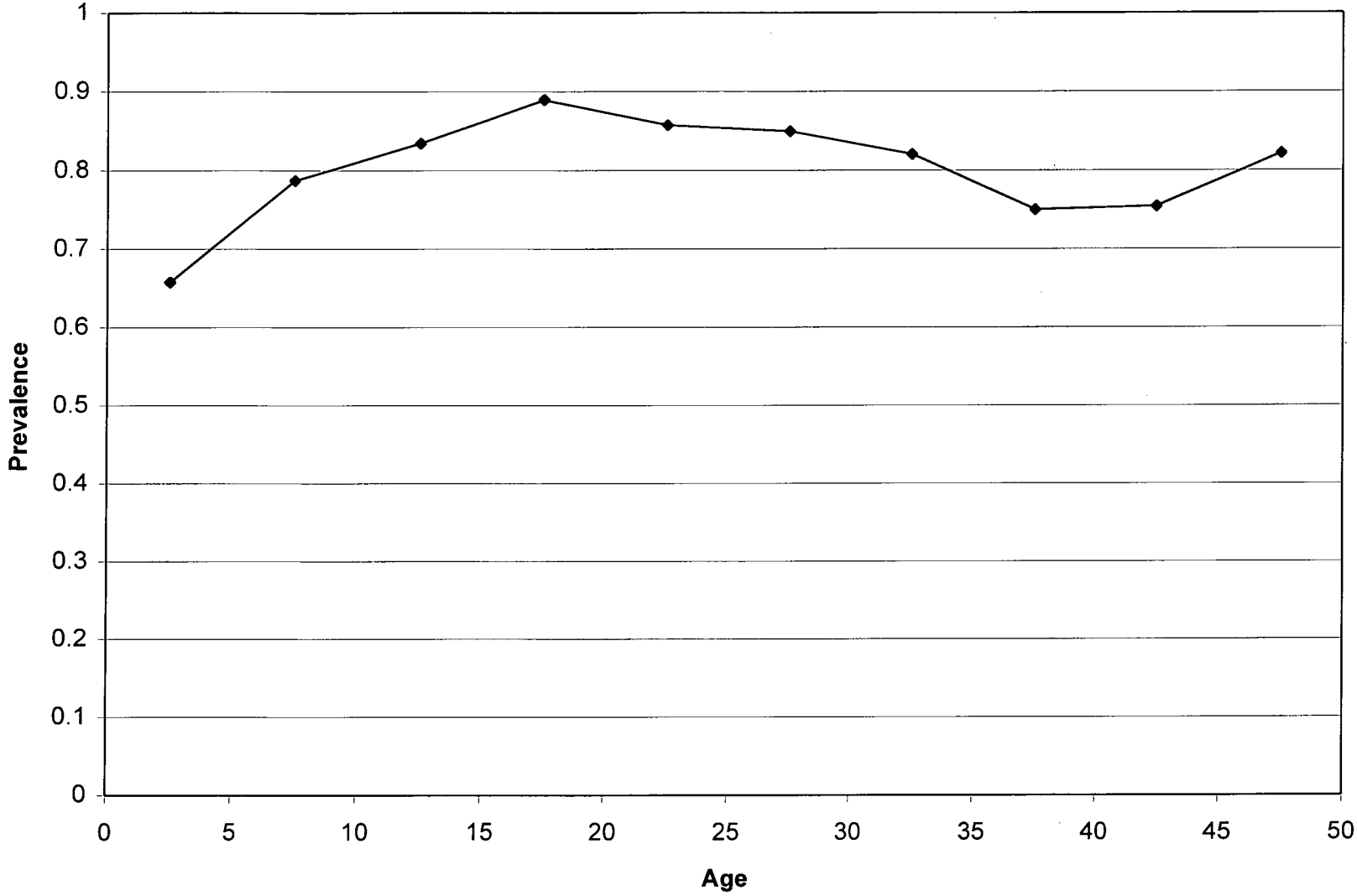

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.2: Prevalence by age of intertriginous freckling.
The curve is based on 3198 NF1 patients from the National Neurofibromatosis Foundation Database.

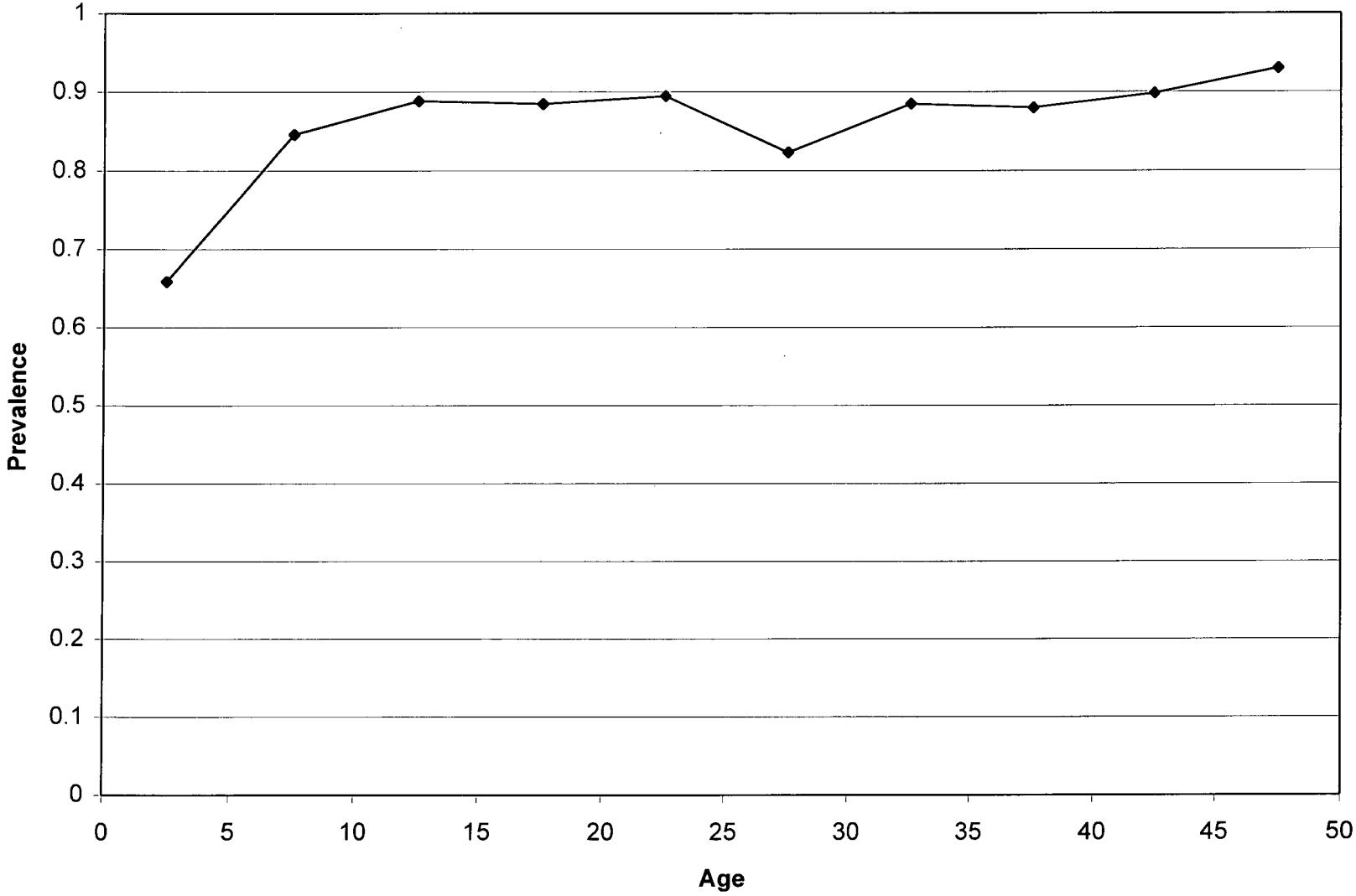

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.3: Prevalence by age of 2 or more subcutaneous neurofibromas. The curve is based on 3255 NF1 patients from the National Neurofibromatosis Foundation Database.

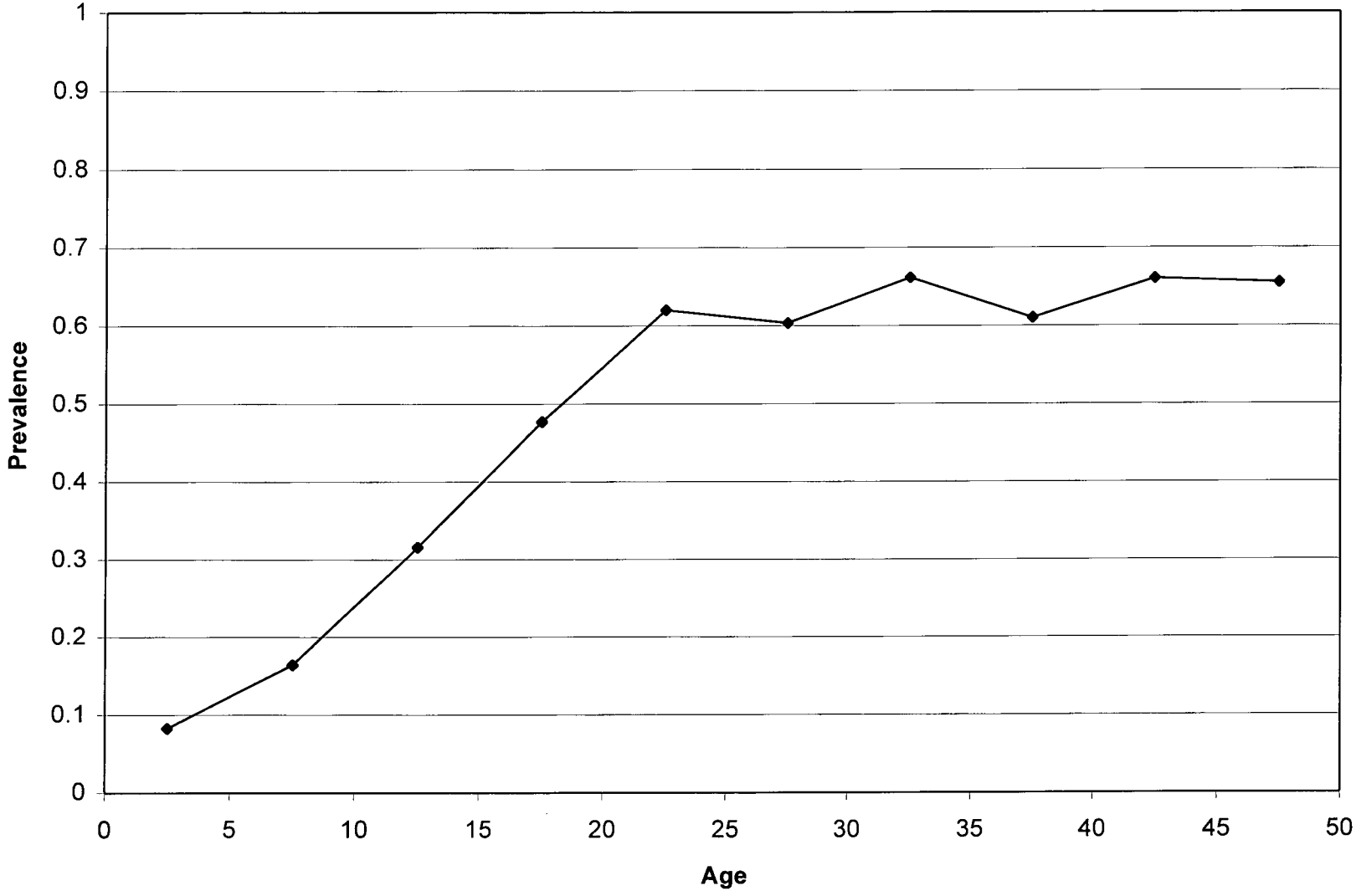

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.4: Prevalence by age of 2 or more cutaneous neurofibromas.
The curve is based on 3283 NF1 patients from the National Neurofibromatosis Foundation Database.

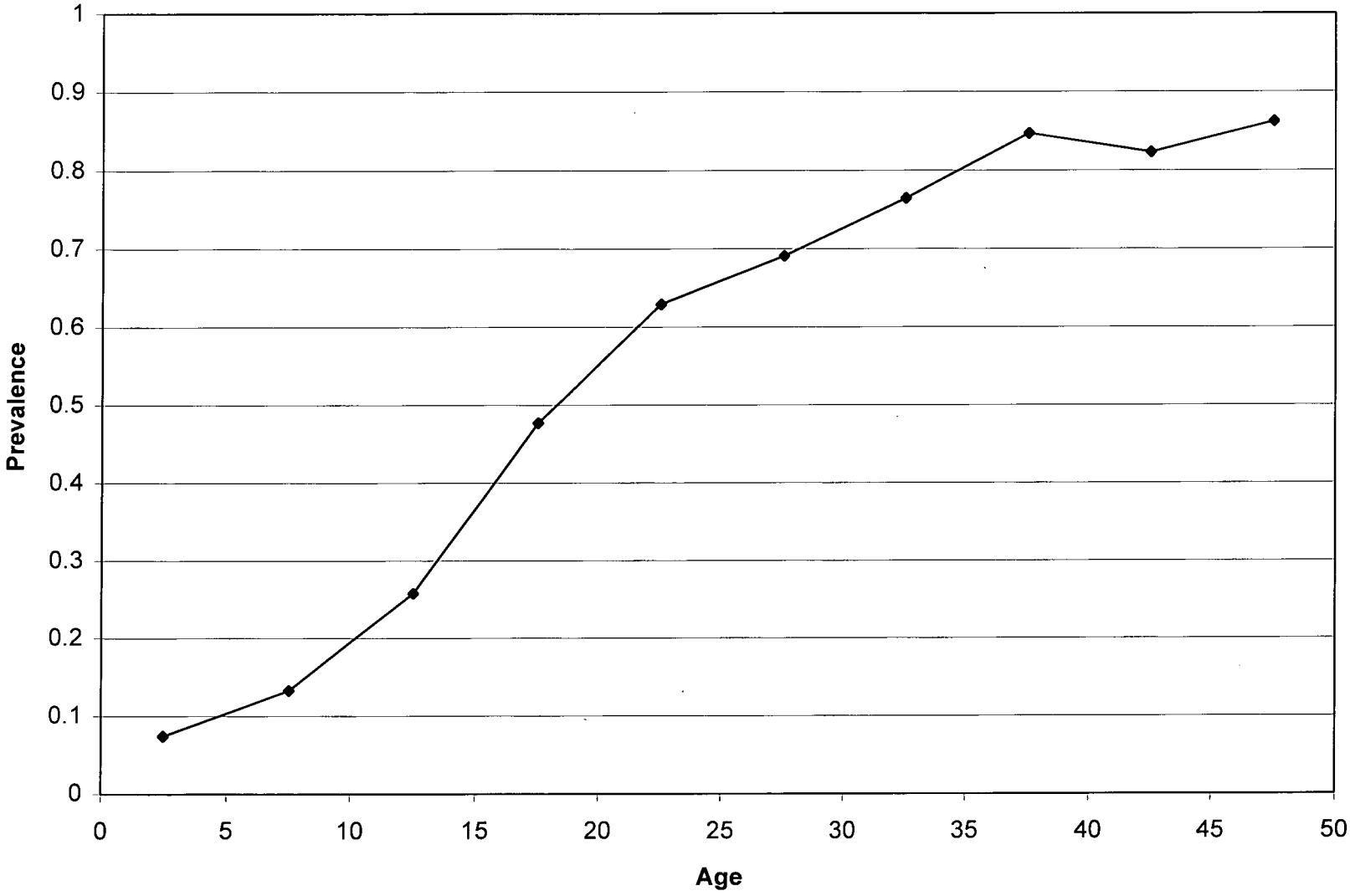

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.5: Prevalence by age of plexiform neurofibromas.
The curve is based on 3283 NF1 patients from the National Neurofibromatosis Foundation Database.

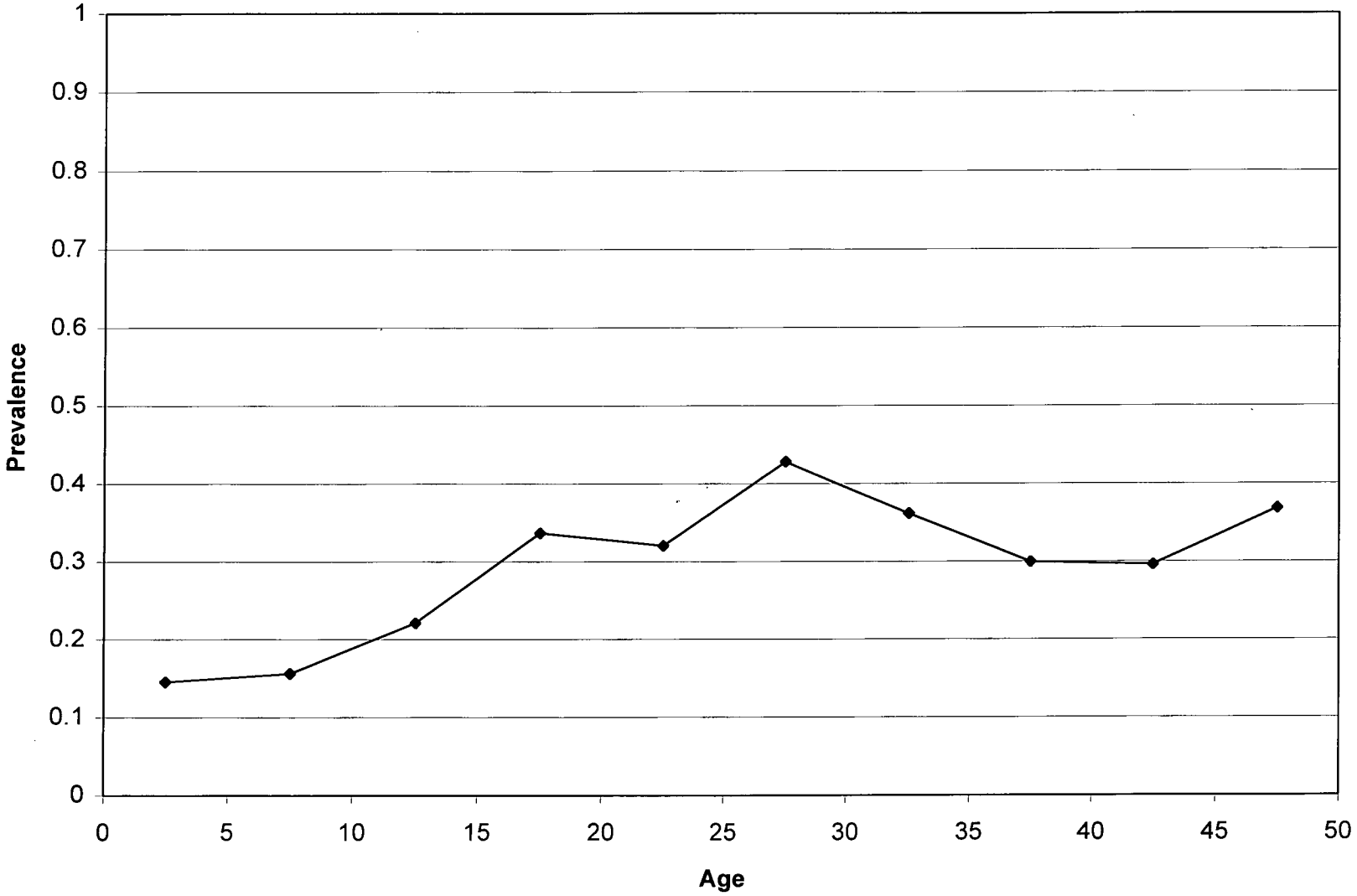

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.6: Prevalence by age of Lisch nodules.
The curve is based on 2432 NF1 patients from the National Neurofibromatosis Foundation Database.

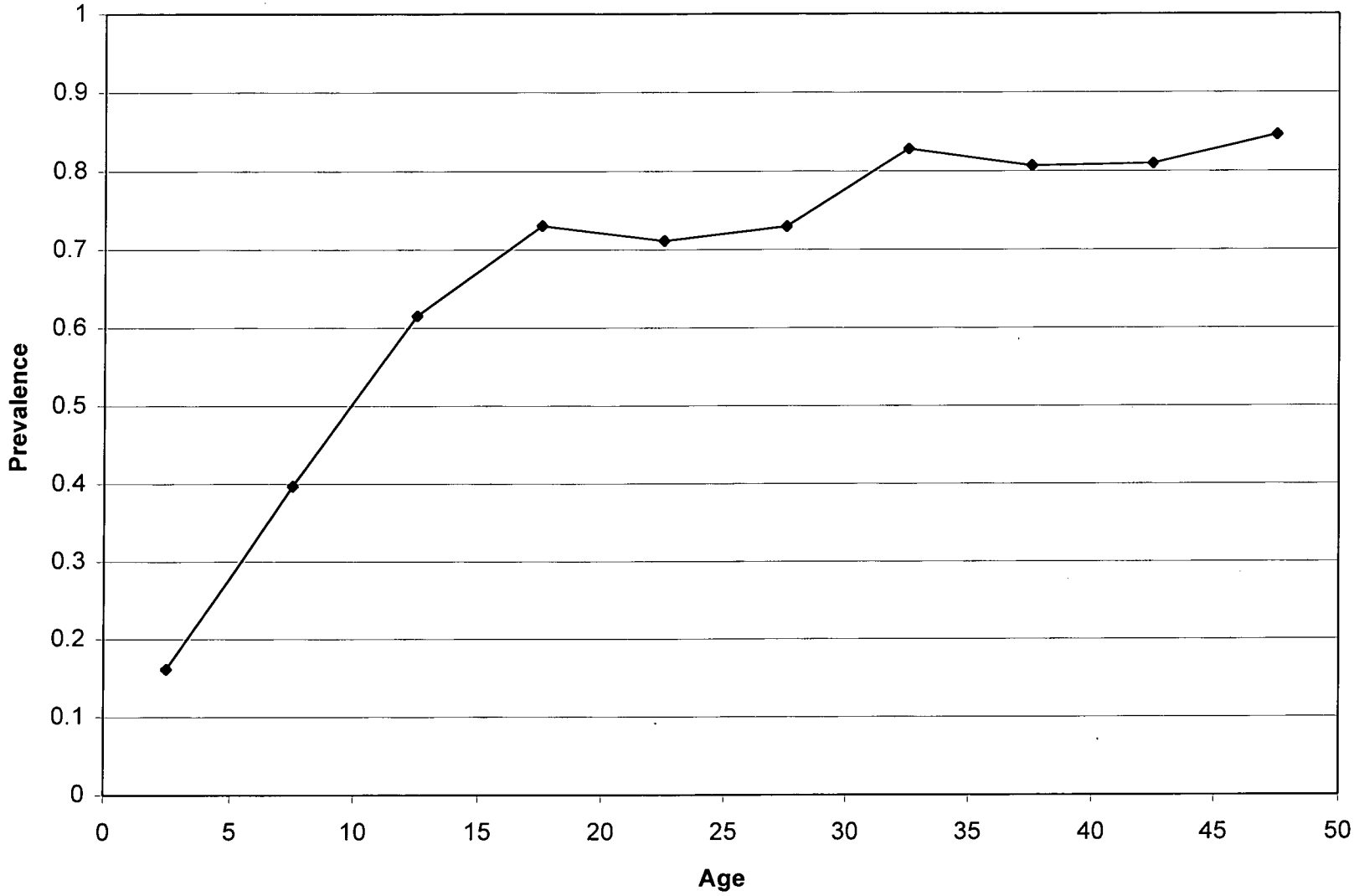

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.7: Prevalence by age of optic glioma.
The curve is based on 1195 NF1 patients from the National Neurofibromatosis Foundation Database.

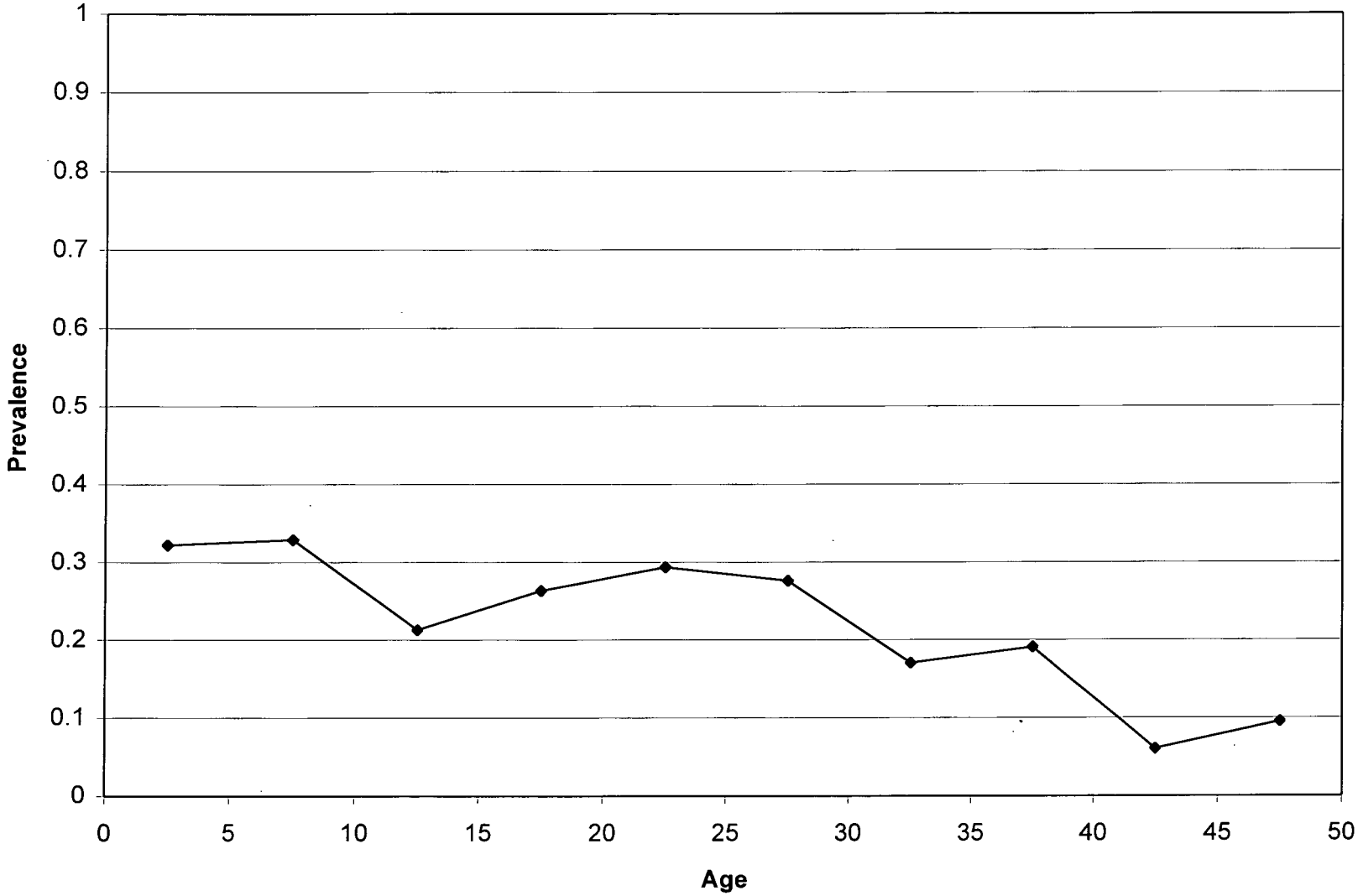

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.8: Prevalence by age of seizures.
The curve is based on among 3308 NF1 patients from the National Neurofibromatosis Foundation Database.

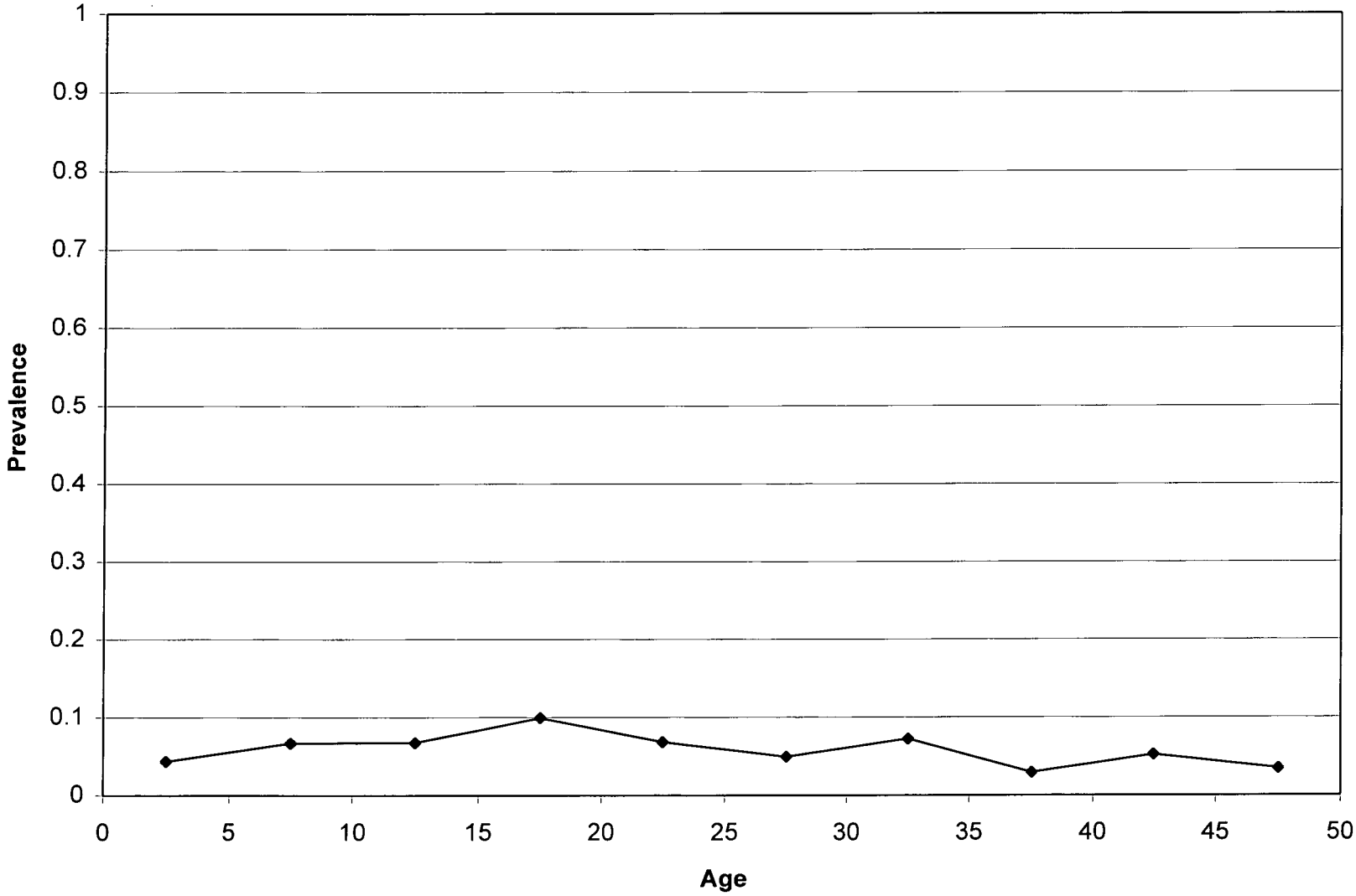

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.9: Prevalence by age of pseudarthrosis.
The curve is based on 3287 NF1 patients from the National Neurofibromatosis Foundation Database.

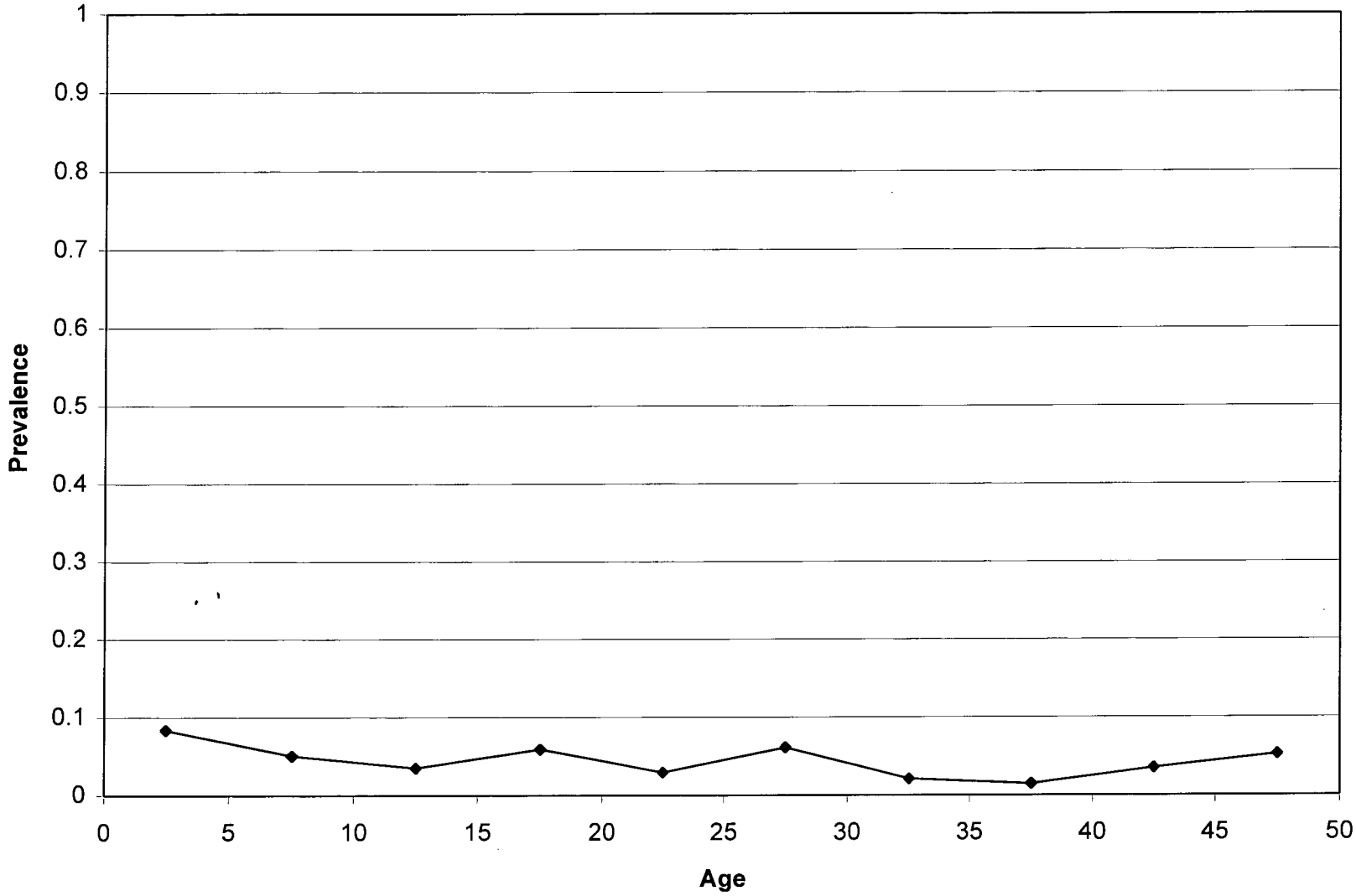

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.10: Prevalence by age of scoliosis.
The curve is based on 3057 NF1 patients from the National Neurofibromatosis Foundation Database.

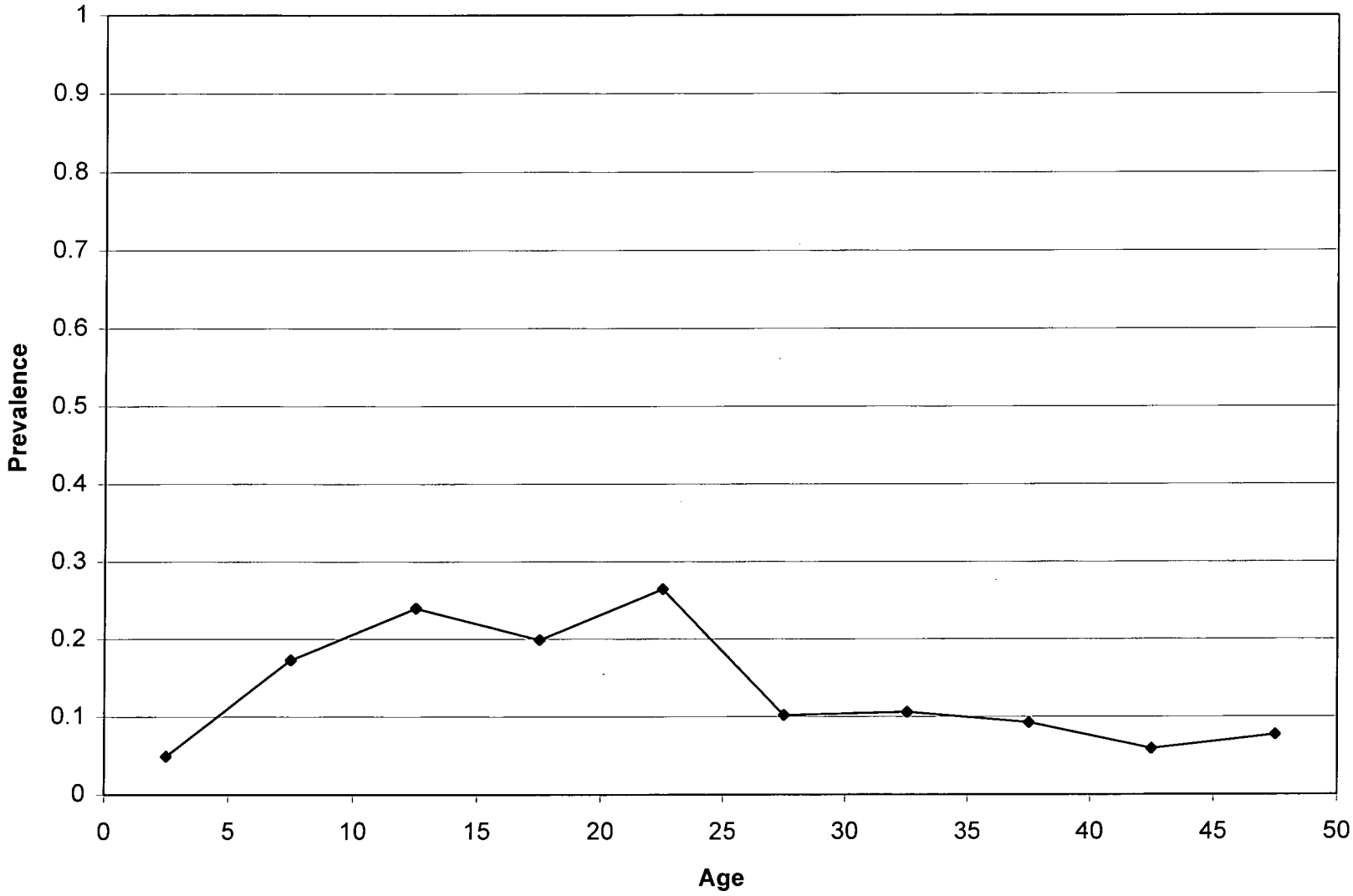

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.11: Prevalence by age of macrocephaly.
The curve is based on 2129 NF1 patients from the National Neurofibromatosis Foundation Database.

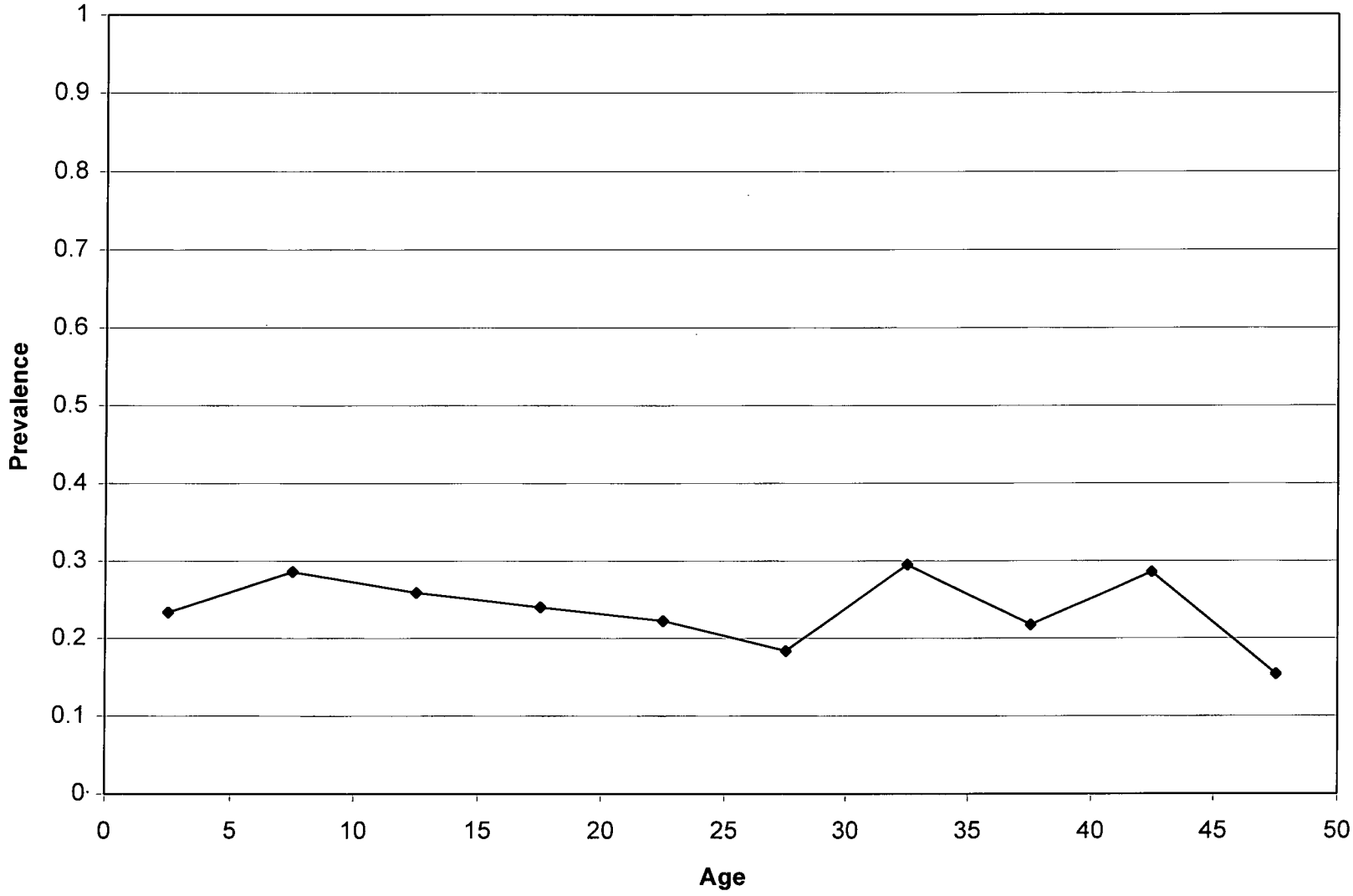

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.12: Prevalence by age of short stature.
The curve is based on 1918 NF1 patients from the National Neurofibromatosis Foundation Database.

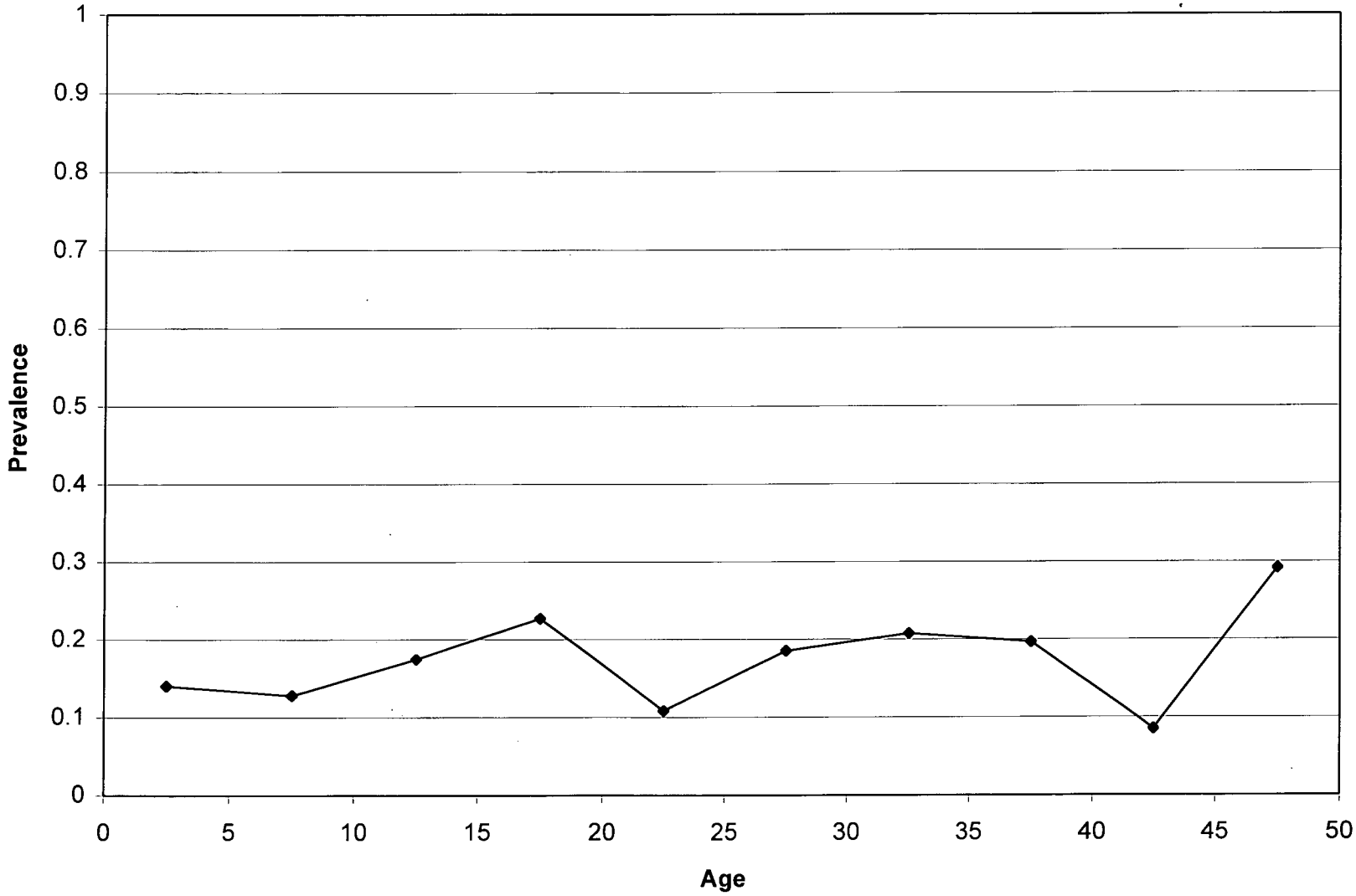
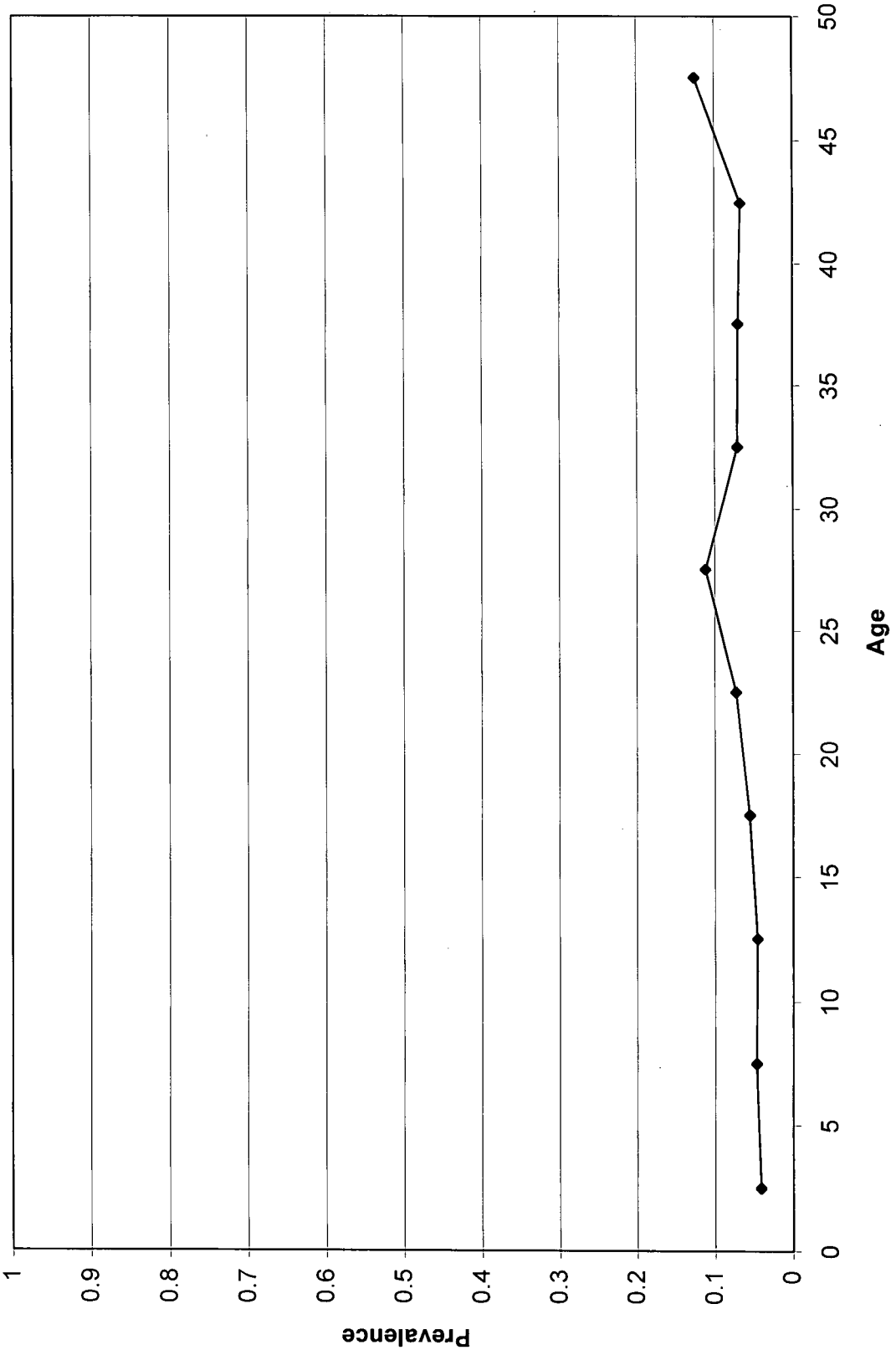

Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

Figure 4.13: Prevalence by age of neoplasms.
The neoplasms do not include optic glioma or neurofibromas. The curve is based on 3129 NF1 patients from the National Neurofibromatosis Foundation Database.


Prevalence
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
40
45
50
Age

4.5.2 Model development and validation

A multi-covariate logistic regressive model was generated for each of 13 different NF1 clinical features, using age (transformed using a log function) and gender as covariates, and each of the 12 other features as possible explanatory variables. Maximum likelihood parameter estimates were used to determine the best fitting model for each of the clinical features in a developmental sample of NF1 probands from the NFDB, and goodness of fit of each model was then evaluated in three other independent samples – an independent "validation" sample of probands from the NFDB, non-proband affected relatives from the NFDB, and the population-based MANF1 sample that includes both probands and non-probands.

The best-fitting models in the developmental sample for the following outcome features had parameter estimates that also had an adequate fit (Hosmer and Lemeshow goodness-of-fit $p \geq 0.05$) in the validation, affected relative and MANF1 samples: intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas, optic glioma, pseudarthrosis, macrocephaly, and other neoplasms (Table 4.1). Models for café-au-lait spots, cutaneous neurofibromas, Lisch nodules, seizures, scoliosis and short stature had an inadequate fit (Hosmer and Lemeshow goodness-of-fit $p<0.05$) in at least one of the samples.

4.5.3 Parameter estimate comparisons

Parameter estimates were independently generated in each of the four samples for the following features: café-au-lait spots, intertriginous freckling, cutaneous

neurofibromas, subcutaneous neurofibromas, plexiform neurofibromas, Lisch nodules, scoliosis and short stature (Table 4.2). Parameter estimates for optic glioma, seizures, pseudarthrosis, macrocephaly and other neoplasms could not be generated in all four samples, due to sparseness of data in at least one of the samples. The corresponding cells in Table 4.2 are blank.

Standard errors are shown for all the parameter estimates in the development subsample (Table 4.1) but not for parameter estimates in the other three subsamples (Table 4.2). Whereas the development subsample was used to identify statistically significant parameter estimates, the latter three subsamples were used to determine which variables had more or less consistent parameter point estimates.

4.5.4 Consistent parameters from validated models

Some of the parameters from models that had an adequate fit (Hosmer and Lemeshow goodness-of-fit $p \geq 0.05$) in all four samples were not consistent when generated independently in each sample. In the plexiform neurofibroma model, the parameter estimates for scoliosis and other neoplasms differed greatly among the four samples. In the pseudarthrosis model, the estimate for freckling was inconsistent. Models from the ill-fit samples differed dramatically often by the estimate of only one parameter. The 10 models that had an adequate fit in at least three of the four samples were recalculated including only variables with consistent parameters. Recalculated parameters for the developmental sample are nearly identical to the initial parameters in Table 4.1 and are summarised as odds ratios with 95% confidence intervals in Table 4.3.

Table 4.1: Logistic regressive models of NF1 features.
Summary of goodness-of-fit and sample size for logistic regressive models of NF1 features. Explanatory features separated by asterisks represent interaction variables. These are the parameters (β's) and their standard errors (SE)for the best fitting model for each feature determined in the development subsample and tested for fit in each of the other subsamples.

Output Feature	Explanatory	β (SE)	Development	Validation	Relatives	MANF1
Café-au-lait spots (CLS)	Freckling	0.25 (.23)	p=0.59	p=0.88	p<0.01	p=0.46
	SNF	-0.63 (.44)	n=987	n=985	n=350	n=180
	Lisch nodules	0.38 (.18)				
	Freckling*SNF	1.12 (.48)				
Freckling	CLS	0.16 (.23)	p=0.88	p=0.23	p=0.20	p=0.41
	SNF	-0.46 (.40)	n=987	n=985	n=350	n=180
	Lisch nodules	0.29 (.20)				
	CLS*SNF	1.31 (.49)				
Cutaneous NFs (CNF)	SNF	0.48 (.15)	p=0.59	p=0.12	p=0.13	p<0.01
	PNF	0.67 (.16)	n=1372	n=1352	n=492	n=281
	Pseudarthrosis	-0.61 (.39)				
Subcutaneous NFs (SNF)	CLS	0.30 (.18)	p=0.16	p=0.06	p=0.07	p=0.47
	CNF	0.69 (.17)	n=1358	n=1331	n=487	n=294
	PNF	0.87 (.21)				
	CNF*PNF	-0.63 (.29)				
Plexiform NFs (PNF)	CNF	0.85 (.22)	p=0.75	p=0.19	p=0.10	p=0.06
	SNF	1.13 (.22)	n=1196	n=1221	n=416	n=250
	Scoliosis	0.62 (.18)				
	Neoplasm	-0.39 (.31)				
	SNF*CNF	-0.71 (.30)				
Lisch nodules	CLS	0.44 (.22)	p=0.74	p=0.63	p=0.50	p=0.01
	CNF	0.67 (.40)	n=969	n=971	n=348	n=172
	Neoplasm	1.74 (.42)				
	CLS*CNF	-0.38 (.43)				

Table 4.1 continued

Output Feature	Explanatory	β (SE)	Development	Validation	Relatives	MANF1
Optic glioma	PNF	0.66 (.37)	p=0.50	p=0.74	p=0.50	p=0.09
	Macrocephaly	0.48 (.31)	n=313	n=328	n=87	n=172
	Neoplasm	1.97 (.48)				
Seizures	SNF	0.10 (.38)	p=0.47	p<0.01	p=0.22	p=0.10
	Neoplasm	2.09 (.42)	n=1300	n=1306	n=473	n=257
	Male gender	0.20 (.33)				
	SNF*Male	0.93 (.49)				
	Neo*Male	-2.05 (.75)				
Pseudarthrosis	Freckling	-0.80 (.31)	p=0.62	p=0.10	p=0.38	p=0.38
	CNF	-0.54 (.35)	n=1270	n=1285	n=461	n=258
	Neoplasm	0.79 (.46)				
	Male gender	0.54 (.27)				
Scoliosis	CNF	-0.75 (.18)	p=0.04	p=0.03	p<0.01	p=0.71
	PNF	0.71 (.18)	n=1289	n=1289	n=447	n=322
Macrocephaly	Lisch nodules	0.45 (.40)	p=0.79	p=0.41	p=0.39	p=0.32
	Optic glioma	1.05 (.45)	n=170	n=190	n=57	n=79
	Short stature	-1.43 (.79)				
	Neoplasm	-2.11 (1.1)				
Short stature	CLS	-0.63 (.26)	p=0.57	p=0.07	p=0.01	p<0.01
	CNF	0.40 (.30)	n=620	n=626	n=261	n=171
	Macrocephaly	-1.21 (.37)				
Neoplasm (other)	Lisch nodules	0.94 (.44)	p=0.93	p=0.07	p=0.92	p=0.39
	Optic glioma	1.93 (.38)	n=411	n=439	n=117	n=141
	Pseudarthrosis	1.76 (.65)				

Table 4.2: Parameter estimates for logistic regressive models of NF1 features.
Summary of parameter estimates for logistic regressive models of NF1 clinical features generated independently in four different subsamples. Explanatory features separated by asterisks represent interaction variables. The models tested include the best fitting model for each feature in the development subsample.

Output Features	Explanatory Features	Development	Validation	Relatives	MANF1
Café-au-lait spots (CLS)	Freckling	0.25	0.31	0.53	0.51
	SNF	-0.63	-0.22	-0.51	-1.46
	Lisch nodules	0.38	0.26	-0.84	-0.50
	Freckling*SNF	1.12	0.60	1.30	2.27
Freckling	CLS	0.16	0.19	0.45	0.69
	SNF	-0.46	-0.63	-0.26	-1.70
	Lisch nodules	0.29	0.17	0.37	1.65
	CLS*SNF	1.31	0.81	1.10	1.98
Cutaneous neurofibromas (CNF)	SNF	0.48	0.69	0.47	1.46
	PNF	0.67	0.96	0.55	0.38
	Pseudarthrosis	-0.61	-0.44	-0.77	2.14
Subcutaneous neurofibromas (SNF)	CLS	0.30	0.23	0.77	0.27
	CNF	0.69	0.92	0.64	2.06
	PNF	0.87	0.74	1.01	0.73
	CNF*PNF	-0.63	-0.58	-0.83	-0.10
Plexiform neurofibromas (PNF)	CNF	0.85	0.79	0.92	0.43
	SNF	1.13	1.20	1.11	0.53
	Scoliosis	0.62	0.32	-0.49	0.55
	Neoplasm	-0.39	-0.22	1.37	-1.48
	SNF*CNF	-0.71	-0.55	-1.01	0.27
Lisch nodules	CLS	0.44	0.50	-0.30	0.63
	CNF	0.67	1.14	1.90	1.19
	Neoplasm	1.74	0.62	0.05	-0.01
	CLS*CNF	-0.38	-0.85	-1.42	-0.20

Table 4.1 continued

Output Features	Explanatory Features	Development	Validation	Relatives	MANF1	
Optic glioma	PNF	0.66	0.50			1.33
	Macrocephaly	0.48	0.48			0.88
	Neoplasm	1.97	1.91			2.37
Seizures	SNF	0.10	0.16			
	Neoplasm	2.09	0.09			
	Male gender	0.20	0.17			
	SNF*Male	0.93	-0.36			
	Neoplasm*Male	-2.05	0.63			
Pseudarthrosis	Freckling	-0.80	-0.33	0.93		
	CNF	-0.54	-0.36	-1.09		
	Neoplasm	0.79	0.35	0.83		
	Male gender	0.54	0.68	0.77		
Scoliosis	CNF	-0.71	-0.43	-1.07		-0.39
	PNF	0.63	0.26	-0.32		0.53
Macrocephaly	Lisch nodules	0.45	1.51			
	Optic glioma	1.05	0.84			
	Short stature	-1.43	-1.40			
	Neoplasm	-2.11	-1.03			
Short stature	CLS	-0.63	-0.39	-0.19		0.65
	CNF	0.40	0.29	0.21		0.20
	Macrocephaly	-1.21	-1.88	-1.94		-0.71
Neoplasm (other)	Lisch nodules	0.94	0.48	1.10		
	Optic glioma	1.93	1.60	1.79		
	Pseudarthrosis	1.76	0.29	3.07		

Some parameters could not be estimated in the smaller samples. The corresponding cells in Table 4.2 are blank.

Table 4.3: Logistic regressive models summarised as odds ratios.
Summary of associations from validated logistic regressive models of NF1 clinical features. These parameters are based on a recalculation of the development subset. Only models with adequate fit and consistent parameter estimates in at least three of the four samples are shown.

Feature	Associated Features	Odds-Ratio (95% C.I.)
Café-au-lait spots (CLS)	Freckling	1.4 (0.8-2.0)
	SNF	0.5 (0.2-1.3)
	Both	*2.3 (1.2-3.7)*
Freckling	CLS	1.2 (0.7-1.9)
	SNF	0.6 (0.3-1.4)
	Lisch nodules	1.3 (0.9-2.0)
	All three	*3.7 (1.8-7.4)*
Cutaneous neurofibromas (CNF)	SNF	1.6 (1.2-2.2)
	Plexiform	2.0 (1.4-2.7)
	Both	*3.2 (2.1-4.7)*
Subcutaneous neurofibromas (SNF)	CLS	1.4 (1.0-1.9)
	CNF	2.0 (1.4-2.8)
	PNF	2.4 (1.6-3.6)
	All three	*3.4 (2.1-5.7)*
Plexiform neurofibromas (PNF)	CNF	2.8 (2.0-4.8)
	SNF	2.5 (1.5-3.6)
	Both	*3.6 (2.3-5.5)*
Lisch Nodules	CLS	1.6 (1.0-2.4)
	CNF	1.7 (0.9-4.3)
	Both	*2.2 (0.4-11.0)*
Optic glioma	PNF	1.9 (0.9-4.0)
	Macrocephaly	1.6 (0.9-2.9)
	Neoplasms	7.1 (2.8-18.1)
	All three	*22.4 (5.8-86.6)*
Pseudarthrosis	CNF	0.6 (0.3-1.2)
	Neoplasm	1.8 (0.9-5.4)
	Male gender	1.6 (1.1-2.9)
	All three	*1.7 (0.7-7.5)*
Macrocephaly	Lisch nodules	1.6 (0.7-3.5)
	Optic glioma	2.9 (1.2-6.9)
	Short stature	0.2 (0.1-1.1)
	Neoplasm	0.1 (0.1-1.1)
	All four	*0.1 (0.1-1.1)*
Neoplasm (other)	Lisch nodules	2.6 (1.1-6.1)
	Optic glioma	6.9 (3.3-14.5)
	Pseudarthrosis	5.8 (1.6-20.9)
	All three	*102 (17.1-616)*

For example, intertriginous freckling was found to be 20% more common (odds ratio = 1.2) in subjects with café-au-lait spots, 40% less common (odds ratio = 0.6) in those with subcutaneous neurofibromas, and 30% more common (odds ratio = 1.3) in those with Lisch nodules. Although only Lisch nodules were significantly associated on their own, freckling was found to be 3.7 times more common in subjects with all three features.

4.6 Discussion

4.6.1 Ascertainment bias

The models I have developed include several associations confirmed in two independent samples of NFDB probands, in their affected relatives and in NF1 patients from the population-based MANF1 sample. The NFDB is comprised of patients seen at specialised clinics, so the development and validation samples of probands are probably more severely affected than the NF1 population in general. The affected relative sample was drawn from the same specialised clinics, but their severity is not as biased as that of the probands (Friedman and Birch 1997b). Nevertheless, since half of NF1 cases represent new mutations, and the NFDB only contains data on 511 affected relatives of 2979 probands, it is likely that many affected relatives of these probands are not included in the NFDB. I expect that affected relatives who are included in the NFDB may be more severely affected than those who were not. In contrast, the MANF1 was collected through genetic registries in north-west England by a limited number of physicians. Both parents were routinely examined. Its ascertainment is nearly 70% and is thought to be representative of the regional NF1 population (McGaughran et al. 1999). Model

parameters that have been confirmed in all four samples are unlikely to reflect database or specialised clinic biases. Instead these models probably reflect trends that exist in the NF1 population at large.

4.6.2 Consideration of binary treatment of variables

Features such as optic glioma, seizures and pseudarthrosis naturally fall into a binary ("present" or "absent") coding scheme, while it might be more informative to treat café-au-lait spots, cutaneous and subcutaneous neurofibromas, scoliosis, macrocephaly, short stature and others as ordinal or continuous variables. Although the NFDB contains ordinal data on many variables, the MANF1 contains mostly binary data. All 13 of the features in this study were treated as binary variables to permit comparison of NFDB models in the MANF1.

4.6.3 Statistical significance

The fit is better for most models (Table 4.1) in the development sample than in any other sample because the parameter estimates were generated in the developmental sample.

Models based on affected relatives from the NFDB and MANF1 samples contain multiple data from families. Standard errors for parameters in these models slightly underestimate true standard errors. Many of the associations in Table 4.3 do not have 95% confidence intervals that exclude 1.0. However, several of these models include three-way interactions (Table 4.2), and the first order parameters must be included to

adhere to the principle of a hierarchically well formulated model (Kleinbaum 1992). Also, a variable can contribute to model fit without being significant itself at $p<0.05$, so the criterion for inclusion in a logistic regressive model was extended to $p<0.2$ (Hosmer and Lemeshow 1989).

4.6.4 Previous reports of associations

Associations involving freckling, Lisch nodules, and plexiform, cutaneous and subcutaneous neurofibromas, are reported in Chapter 2 as pair-wise associations of weak magnitude. For example, freckling and Lisch nodules were shown to have a pair-wise age-stratified odds ratio of 1.8 (95% C.I.=1.3-2.4). This chapter shows that most of these associations not only persist when controlling for age and other common NF1 features, but increase slightly in strength when the presence of multiple features is considered. The presence of café-au-lait spots and subcutaneous neurofibromas as well as Lisch nodules make freckling 3.7 (95% C.I.=1.8-7.4) times more likely. Furthermore, this chapter shows that these pair-wise associations exist simultaneously. For example, cutaneous and subcutaneous neurofibromas are both significantly associated with plexiform neurofibromas (Table 4.3).

The pair-wise association between optic glioma and neoplasms has also been previously reported with an odds ratio of 5.8 (Friedman and Birch 1997a) but gains even more strength when other features are taken into consideration. Optic glioma is 22.4 (95% C.I.= 5.8-86.6) times more common when plexiform neurofibromas and macrocephaly, as well as neoplasms, are present and age is taken into consideration.

4.6.5 Pathogenetic interpretation of associations

Many of the associations I observed were non-reciprocal – only one of a pair of features appears in the other's model. This suggests that the two features were not of primary importance in accounting for each others' status. I also observed several reciprocal associations. As a conceptual framework to understand the interdependencies of clinical features of NF1, it is not easy to pool the results of 13 separate models. Numerical taxonomy and log-linear models are easier to interpret, but they are more susceptible to confounding by age and do not provide initial parameters for the final study in this thesis, multivariate analyses (see Chapter 7). Still, a few features appeared to be closely associated (see below). In general, features were considered to be closely associated if each feature appeared as an explanatory variable with a positive parameter estimate in each of the other group members' models. Such a relationship means that these features must be taken into account to accurately describe the occurrence of other features belonging to the same group. Fundamental pathogenetic differences may exist between subjects who have one or more of a group's features and those who do not, and the mechanisms shared by associated features may be different for each group of features. However, these NF1 features are not mutually exclusive, and many patients belong to more than one group.

1) Café-au-lait spots, intertriginous freckles and Lisch nodules are all derived from cells of melanocytic origin (Weston 1981; Perry and Font 1982). Café-au-lait spots contain melanosomes with giant pigment particles. Intertriginous freckles develop through a process that does not require light exposure, but they too involve pigment and darken with sun exposure (Fitzpatrick 1981). Histologically, Lisch nodules are

melanocytic hamartomas. Associations between Lisch nodules and pigmentary features have been previously reported (Pietruschka 1961; Zehavi et al. 1986), but the responsible mechanism is unknown.

2) The associations observed between the occurrence of plexiform, cutaneous and subcutaneous neurofibromas are consistent with the histopathological similarity between these lesions (Harkin and Reed 1969; Burger and Scheithauer 1994). In addition, each type of neurofibroma is associated with acquired loss or mutation of the normal NF1 allele in at least some cases (Sawada et al. 1996; Serra et al. 1997). The negative 3-way interaction terms in two of the three models suggest that associations involving neurofibromas are not additive.

The association between subcutaneous neurofibromas and café-au-lait spots is negative in the café-au-lait spot model, but positive in the subcutaneous neurofibroma model (Table 4.1). This is because the coefficient for subcutaneous neurofibromas in the café-au-lait spot model changed from positive to negative after adding the interaction term. Similarly, the coefficient for subcutaneous neurofibromas in the intertriginous freckling model changed from positive to negative after adding the interaction term between café-au-lait spots and subcutaneous neurofibromas, indicating a positive three-way interaction. Melanocytes in café-au-lait spots and intertriginous freckles and Schwann cells in subcutaneous neurofibromas are derived from the embryonic neural crest (Weston 1981). This is consistent with the suggestion that NF1 is a neurocristopathy (Huson and Hughes 1994) but does not explain why these and not other neural crest-derived tissues are involved in NF1 and why many features of NF1 do not appear to be abnormalities of neural-crest derived tissues (see section 2.6.1).

3) The common thread between optic glioma, other neoplasms and macrocephaly could be glial hyperplasia resulting from haploinsufficiency of neurofibromin. Most of the other neoplasms in patients in this study involve the central nervous system and most of these are gliomas (Friedman and Birch 1997a). Patients with hydrocephalus and plexiform neurofibromas on the head were excluded from the analyses of head circumference, so enlargement of the head in the remaining patients must be due to enlargement of the scalp, skull or brain. In NF1, enlargement of the brain is the likely cause (Riccardi 1992; Huson 1994). Gutmann et al. (1999) have directly demonstrated an effect of NF1 haploinsufficiency on glial cell proliferation.

4.6.6 Cross-sectional nature of data

While these models are accurate descriptors of feature occurrence, they cannot be used to predict which patients will get what features. The NFDB data are largely cross-sectional, with 74% of the subjects seen only once. The MANF1 is exclusively cross-sectional. A fitted logistic regressive model can be used to predict the risk for an individual developing a particular feature in follow-up studies, but not in cross-sectional studies such as this one (Kleinbaum 1992). Currently available longitudinal clinical data in NF1 are too limited in number of subjects and duration of study for this purpose; large-scale longitudinal studies of the natural history of NF1 would be necessary to develop predictive models.

4.7 Conclusion

The occurrence of NF1 clinical features can be described to some extent by taking other disease features and age into account. These associations have been demonstrated in probands, their affected relatives and in a completely independent population-based sample. This suggests that the associations are true of NF1 patients in specialised clinics and of NF1 patients at large. Phenotypic studies of affected relatives can determine the importance of familial and genetic factors in the development of these common NF1 features. The models developed here identify and quantify the covariates that must be taken into account in familial studies in Chapter 7 .

5. UNIDENTIFIED BRIGHT OBJECTS ON MRI ASSOCIATED WITH DIAGNOSTIC FEATURES OF NEUROFIBROMATOSIS 1

5.1 Hypothesis

Unidentified bright objects (UBOs) do not occur independently of other neurofibromatosis 1 (NF1) features.

5.2 Objective

Use logistic regressive models to identify individual features whose occurrence is associated with the occurrence of UBOs in NF1 patients.

5.3 Introduction

"Unidentified bright objects" (UBOs) have been observed on MRI in 43-93% of children with neurofibromatosis 1 (NF1) (Bognanno et al. 1988; Griffiths et al. 1999). UBOs are areas of increased image brightness that can be visualised only under particular scanning configurations (Truhan and Filipek 1993). They show no mass effect or contrast enhancement (Sevick et al. 1992). Pathologically, they correspond to areas of vacuolar or spongiotic change in the brain substance and may represent increased fluid within the myelin associated with hyperplastic or dysplastic glial proliferation (DiPaolo et al. 1995). UBOs evolve over time and are almost never seen in patients over the age of 20 years (Sevick et al. 1992; DiMario and Ramsby 1998). A correlation between the location of these lesions and IQ score has been suggested, but no consistent relationship has been found between the learning disabilities that frequently occur in NF1 patients and the presence of UBOs (North 1999). I show here that UBOs may also be associated with the occurrence of other clinical features in young (<21 years) NF1 patients.

5.4 Subjects and methods

5.4.1 Subjects

523 patients between the ages of 2 and 20 years who met the NIH Diagnostic Criteria for NF1 and had cranial MRI examinations were selected from the National NF Foundation International Database (NFDB) (Friedman et al. 1993). The presence or absence of the following NIH Diagnostic features was known in each patient: ≥6 café-au-lait spots of sufficient size for age, intertriginous freckling, ≥2 Lisch nodules, ≥2 neurofibromas or one plexiform neurofibroma, a typical bony lesion and optic glioma. Although an affected first-degree relative is also one of the NIH Diagnostic Criteria and accounts for the fact that some of the patients included had only one diagnostic feature of NF1, family history was not considered in this analysis.

5.4.2 Statistical analysis

The presence or absence of UBOs on cranial MRI examination was determined for each patient (DeBella et al. 2000a). The presence or absence of each of the six diagnostic features and of CNS neoplasms (other than optic glioma) was determined during the same clinical visit (Gutmann et al. 1997). The χ^2 and Cochran-Armitage trend statistics were used to test whether the presence of UBOs was independent of the number of diagnostic features. The Cochran-Armitage test partitions the χ^2 into component parts – χ^2 for linear trend and χ^2 for departure from linear trend (Zar 1999). Logistic regression was used to quantify the associations between UBOs and each of the diagnostic features simultaneously, while controlling for age and gender (SAS 1996). Features with $p>0.10$

in the first model were excluded from subsequent models. Parameters for the remaining features were re-estimated and second order interactions were evaluated.

5.5 Results

5.5.1 Frequency of clinical features analysed

259 (50%) of the 523 NF1 patients from the National Neurofibromatosis Foundation International Database (NFDB) under 21 years of age who had undergone head MRI had UBOs. 492 (94%) had ≥6 café-au-lait spots, 426 (81%) intertriginous or axillary freckling, 253 (48%) Lisch nodules, 128 (24%) ≥2 subcutaneous neurofibromas, 85 (16%) ≥2 cutaneous neurofibromas, 100 (19%) ≥1 plexiform neurofibromas, 73 (14%) a typical bony lesion, 70 (13%) had optic glioma – 23 (4%) symptomatic and 47 (9%) asymptomatic. 11 (2%) patients had other CNS neoplasms – 7 non-optic gliomas, 1 ependymoma, 1 ganglioneuroma, 1 hamartoma of the pons and midbrain, and 1 hypothalamic neoplasm. Figure 5.1 shows the frequency of UBOs by age.

5.5.2 Unidentified bright objects by number of diagnostic features

Figure 5.2 shows the frequency of UBOs among 523 NF1 patients from the NFDB 2-20 years of age according to the number of diagnostic features present in each patient. The frequency of UBOs in my sample does not increase with age in this range (Figure 5.1) but increases from 29% in patients who have only one diagnostic feature to 100% in patients who have six features (χ^2=28.0, p<.0001; Cochran-Armitage Trend = -5.201, p<.001). The mean age of patients with 1 or 2 diagnostic features was 8 years

Figure 5.1: Prevalence by age of Unidentified Bright Objects.
The curve is based on 523 neurofibromatosis 1 (NF1) patients from the National Neurofibromatosis Foundation International Database who had cranial MRI. Dotted lines indicate 95% confidence intervals.

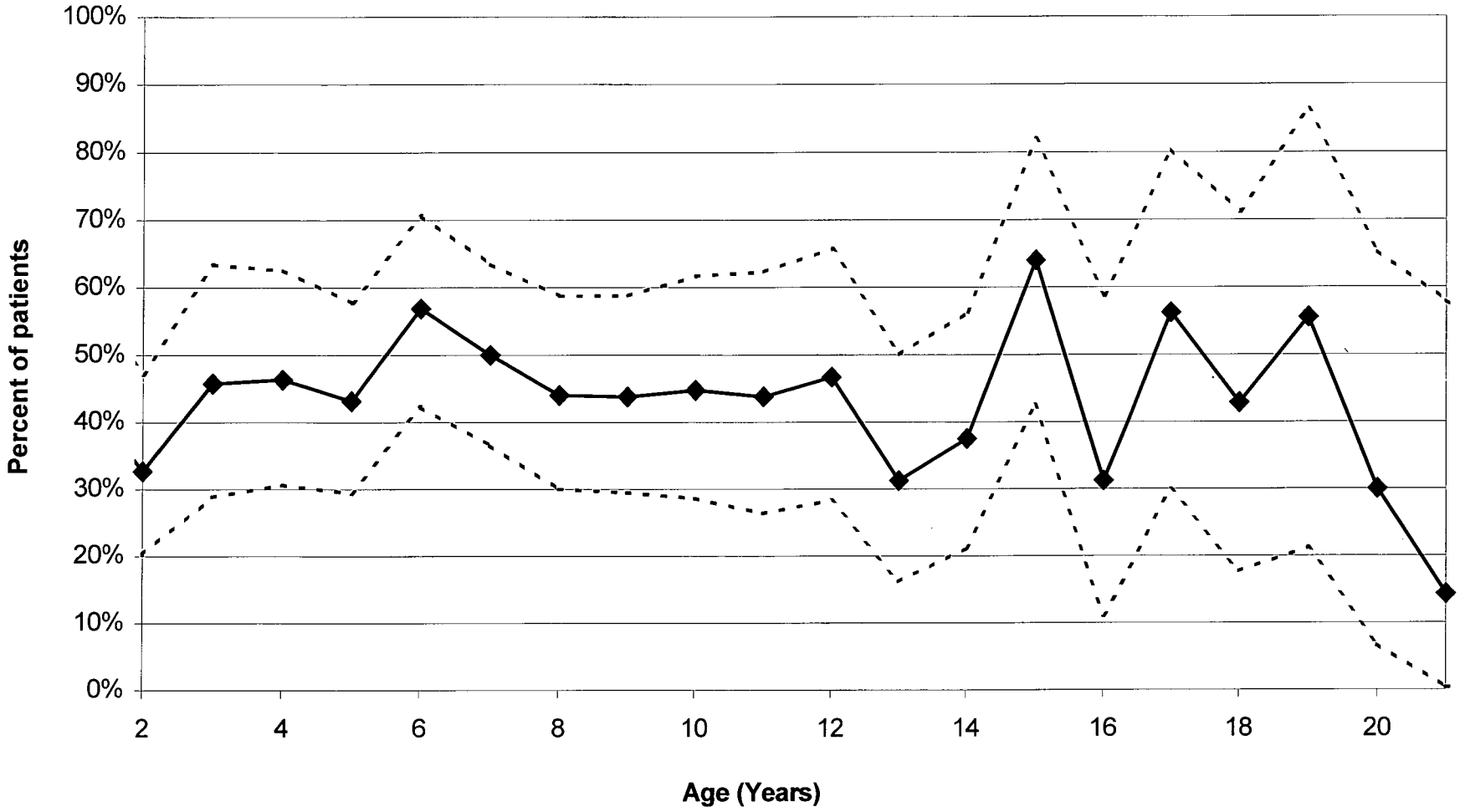
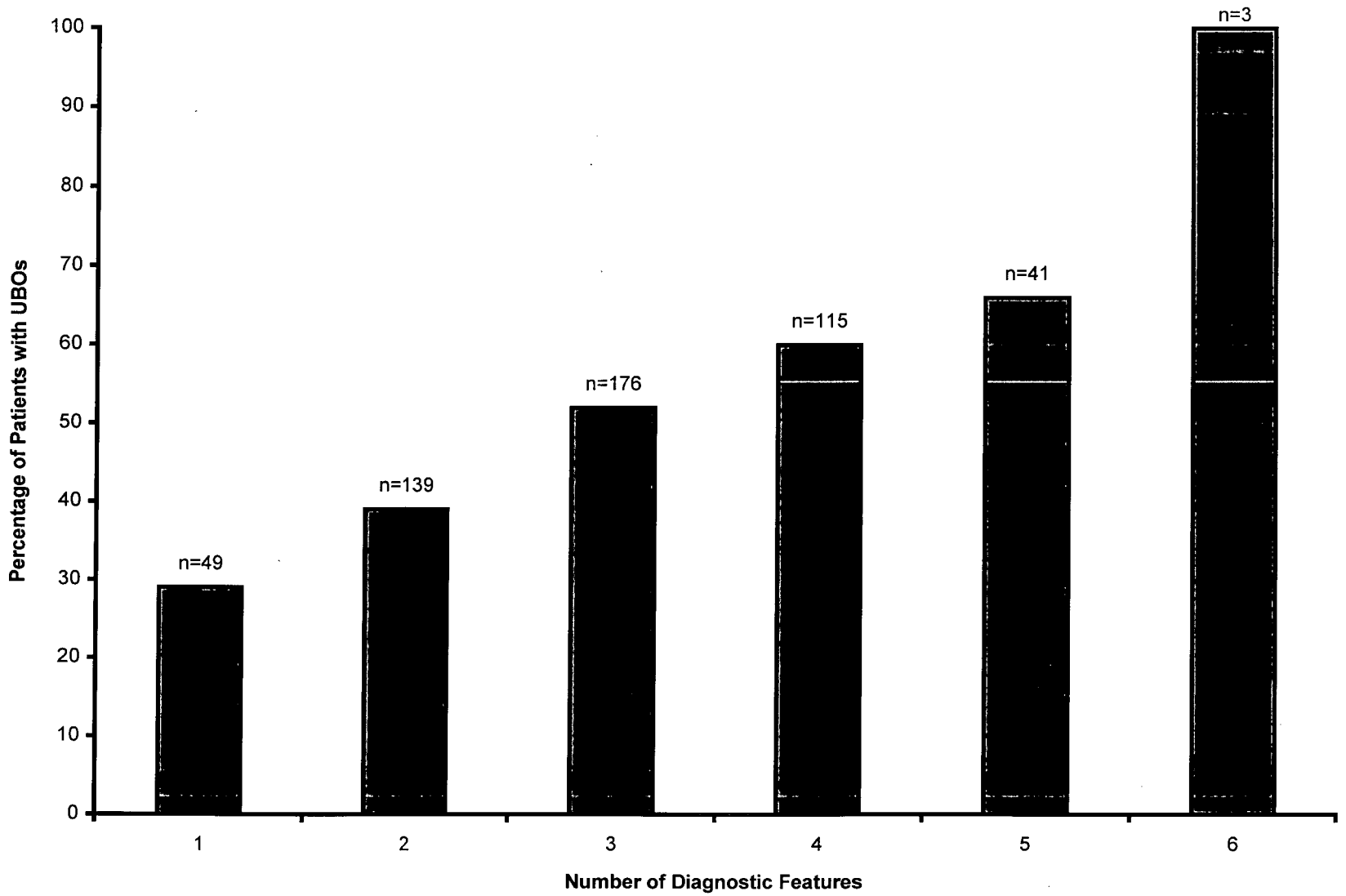

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Percent of patients
2
4
6
8
10
12
14
16
18
20
Age (Years)

Figure 5.2: Unidentified Bright Objects by the number of diagnostic features. Percentage of Unidentified Bright Objects on MRI by the number of diagnostic features in 523 NF1 patients between 2 and 20 years of age from the National Neurofibromatosis Foundation International Database. The label above each column indicates the number of patients with the respective number of diagnostic features.


Percentage of Patients with UBOs
100
90
80
70
60
50
40
30
20
10
0
n=49
n=139
n=176
n=115
n=41
n=3
1
2
3
4
5
6
Number of Diagnostic Features

and the mean age of those with 4, 5 or 6 diagnostic features was 12 years.

5.5.3 Logistic regressive model of unidentified bright objects versus diagnostic features

Table 5.1 summarises the associations between UBOs and other clinical features estimated by multi-covariate logistic regression. Model 1 suggests that café-au-lait spots, freckling, cutaneous and plexiform neurofibromas and characteristic bony lesions are not associated with UBOs. These variables were excluded and the remaining associations recalculated in Model 2. Model 2 suggests that significant associations exist among NF1 patients between UBOs and the presence of Lisch nodules, subcutaneous neurofibromas, optic gliomas and neoplasms, but not age or gender. None of the second order interactions was significant.

5.6 Discussion

5.6.1 Unidentified bright object associations

I found UBOs to be associated with the number of diagnostic features in young NF1 patients (Figure 5.2), but most significantly with optic gliomas, other CNS neoplasms, subcutaneous neurofibromas and Lisch nodules (Table 5.1). The percentage of patients with Lisch nodules and neurofibromas increases with age (DeBella et al. 2000b). However, my approach adjusts for age (and gender), so the observed associations are not confounded by age and are not an artefact of age-specific sampling. The frequencies of these features in NFDB patients are similar to those reported for other patients in this age group (Griffiths et al. 1999; McGaughran et al. 1999).

Table 5.1: Features associated with Unidentified Bright Objects.
Associations of diagnostic features with the presence of Unidentified Bright Objects in 523 NF1 patients. Logistic regression was used to quantify the associations between UBOs and each of the diagnostic features simultaneously, while controlling for age and sex. Age, gender and features with $p<0.10$ in Model 1 were included in Model 2. There were no significant second order interactions between the explanatory features in Model 2.

	Model 1	**Model 2**		
Main Effects	*P-Value*	*P-Value*	*Odds Ratio*	*95% Confidence Interval*
Age	0.83	0.75	0.99	0.95-1.03
Male Gender	0.10	0.10	0.74	0.52-1.05
Café-au-Lait Spots	0.18	—	—	—
Freckling	0.32	—	—	—
Lisch Nodules	0.04	0.02	1.59	1.10-2.32
Neurofibromas				
Cutaneous	0.95	—	—	—
Subcutaneous	0.004	0.002	2.00	1.30-3.08
Plexiform	0.18	—	—	—
Bony Lesion	0.59	—	—	—
Optic Glioma	0.01	0.009	2.08	1.20-3.62
Other CNS Neoplasms	0.04	0.04	8.99	1.13-69.97

5.6.2 Bias

The patients in this study were selected from the NFDB because they had cranial MRI. Most of the centres that contribute data to the NFDB do not routinely do MRI examinations on all young NF1 patients, in accordance with current recommendations (Gutmann et al. 1997). I did not know why the patients in this study underwent MRI. Therefore, I was concerned that the patients who did have MRIs were more likely to have symptoms of intracranial pathology. All patients in this study underwent MRI examinations, but the frequency of such intracranial lesions as optic and other CNS gliomas was not unusually high compared to a prospective (Griffiths et al. 1999) and a population-based (McGaughran et al. 1999) studies. Nevertheless, an inclusion bias has to be considered. MRIs on patients in my study were interpreted by a different radiologist at each centre. Most of the patients in my study were seen at pediatric clinics (Friedman et al. 1993) and agreement between different pediatric radiologists with respect to the presence or absence of UBOs on MRIs of NF1 patients has been shown to be around 85% (DeBella et al. 2000a). Therefore, I expect the criteria for defining UBOs and differentiating them from initial low-grade gliomas or other brain lesions to be less consistent than if all patients had been examined by the same radiologist.

Patient information was not kept from the radiologists who interpreted the MRI scans, and they may have been more likely to recognise UBOs if a diagnosis of NF1 had already been established or was obvious on clinical or radiological examination. This is unlikely to explain the association observed between the occurrence of UBOs and such non-prominent features as Lisch nodules. Moreover, I found no statistically significant

association between UBOs and two of the most obvious diagnostic features – café-au-lait spots and cutaneous neurofibromas.

5.6.3 Cross-sectional nature of data

A recent longitudinal MRI study by Griffiths et al. (1999) found brain tumours in several children with NF1 that developed at the site of a previously-recognised UBOs. In addition, the children who developed brain tumours tended to have more UBOs than other children their age with NF1. I did not analyse the precise location or number of UBOs in these patients, and my data are cross-sectional, not longitudinal. Also, follow-up may help define some of the lesions labelled as UBOs. Nevertheless, I did observe a strong association between the occurrence of UBOs and CNS gliomas.

The cross-sectional nature of my data preclude conclusions about whether UBOs develop before or after the other associated features in individual NF1 patients. It would be important to assess this prospectively because UBOs are more prevalent at a younger age than Lisch nodules, subcutaneous neurofibromas and neoplasms (Friedman and Riccardi 1999; DeBella et al. 2000a; DeBella et al. 2000b) and might predict the future development of other features in young NF1 patients. For this reason they have been proposed as an additional diagnostic criterion in children (Curless et al. 1998). UBOs may turn out to be a reliable diagnostic criterion, but not before they are better defined in terms of location, size and number, and their sensitivity and specificity are properly studied (DeBella et al. 2000a).

5.6.4 Pathogenesis

My observations support a pathogenetic relationship between UBOs and certain other features in young NF1 patients. The common thread between the associated features (optic gliomas, other neoplasms, Lisch nodules, and subcutaneous neurofibromas) may be dysregulated cellular proliferation resulting from haploinsufficiency of neurofibromin (Gutmann et al. 1999; Ingram et al. 2000), but it is hard to understand what this has to do with UBOs if they are simply areas in which the myelin is immature and contains increased fluid. The key may be underlying hyperplastic or dysplastic glial cell proliferation (DiPaolo et al. 1995), which leads to formation of the altered myelin in UBOs. If widespread dysregulation of glial cell proliferation in the brains of young NF1 patients is involved in the pathogenesis of UBOs, the mechanism could be similar to that underlying the formation of optic and other CNS gliomas (Friedman and Birch 1997a), and, by analogy, to the formation of Lisch nodules and subcutaneous neurofibromas in these patients.

5.7 Conclusion

UBOs and other NF1 clinical features do not occur independently and may be pathogenetically related.

6. ANALYSIS OF LOCAL AND FAMILIAL FACTORS IN NEUROFIBROMATOSIS 1 LESIONS

6.1 Hypothesis

The development of café-au-lait spots, plexiform neurofibromas and cutaneous neurofibromas is influenced by both local and familial factors.

6.2 Objective

To test for and estimate associations within body segments and within families for the occurrence of café-au-lait spots, plexiform neurofibromas and cutaneous neurofibromas.

6.3 Introduction

The defining feature of neurofibromatosis is the neurofibroma: a complex benign tumour arising in the fascicles of peripheral nerves (see section 1.5). Café-au-lait spots are another cardinal pathologic feature of NF1. They are present soon after birth in almost all NF1 patients, and their number and size tend to increase during the first decade of life (Riccardi 1982).

It has been hypothesised that histamine or other products secreted by mast cells may influence the growth of neurofibromas (Giorno et al. 1989; Riccardi 1992). A neurofibroma that contains an excess of mast cells could stimulate not only its own growth, but that of other neurofibromas nearby. Secreted factor may also be triggered by local trauma. Freckles and other areas of hyperpigmentation tend to occur in skin folds, presumably due to local environmental factors (Fitzpatrick 1981; Riccardi 1992). Cutaneous neurofibromas may arise in an area that has been injured (Riccardi 1990).

I have shown in Chapters 2 and 4 that individuals with diffuse plexiform neurofibromas are more likely also to have cutaneous neurofibromas and those with café-au-lait spots are more likely to have subcutaneous neurofibromas. Also, the occurrence of several NF1 clinical features was found to be associated in affected parents and children: Lisch nodules, optic glioma, learning disability or mental retardation, macrocephaly and short stature. Here I test the hypotheses that the development of these lesions may be influenced by local or familial factors.

6.4 Subjects and methods

6.4.1 Subjects

547 NF1 patients, including 117 affected individuals in 52 families, were selected from the NF Institute database (Riccardi 1992). All of these patients were evaluated between 1979 and 1995 by Dr. Vincent Riccardi, and all meet the NIH diagnostic criteria for NF1 (NIH 1988; Gutmann et al. 1997). For each patient, the presence of 1 or more café au lait spots, 1 or more cutaneous neurofibromas, and 1 or more diffuse plexiform neurofibromas was recorded for each of the ten divisions of the body surface shown in Figure 6.1.

6.4.2 Analysis of local effect

I used two-layered Mantel-Haenszel tests (see section 2.4.3) to look for local associations between the presence of diffuse plexiform neurofibromas and cutaneous neurofibromas in individual body segments of each NF1 patient (SPSS 1998). I stratified

simultaneously by the body segment being considered and by the number of other body segments with 1 or more cutaneous neurofibromas (a categorical variable with range 0 to 9). This stratification was used to adjust for the fact that an NF1 patient who has a greater total number of body segments with 1 or more neurofibromas is more likely to have at least one neurofibroma in any particular segment than an NF1 patient who has fewer total body segments affected. Confidence intervals for the summary odds-ratios were obtained using a jack-knife based on 20 different subgroups –sufficient to get stability in the estimate. The jack-knife involved calculating the standard error an additional 20 times, each time excluding a different subgroup. The mean and variance of these 20 additional standard errors were then used to estimate the true standard error (Miller 1974). Odds-ratio homogeneity was assessed using the Breslow-Day test (SPSS 1998). Local associations between café-au-lait spots and cutaneous neurofibromas and between café-au-lait spots and plexiform neurofibromas were also analysed in this manner.

6.4.3 Skin surface area

The body divisions used in this study cover varying amounts of skin surface area, so I checked for an association between segment surface area and the presence of ≥1 cutaneous neurofibroma. Using logistic regression, I set the segment area as the independent variable and the presence or absence of cutaneous neurofibromas as the dependent variable. I also checked for an association between surface area and prevalence of diffuse plexiform neurofibromas. Since the median age of my patients was 12 years, I approximated the surface area percentages of the body divisions by taking the

mean of the values cited for children and for adults (Palehorse 1997). The proportions of total surface area assigned to each body segment were: head=9%, neck=3%, right upper torso=9%, left upper torso=9%, right lower torso=9%, left lower torso=9%, right arm=9%, left arm=9%, right leg=17%, and left leg=17%.

6.4.4 Total number of neurofibromas

In addition to the data on whether each body segment was affected by 1 or more cutaneous neurofibromas, complete counts of cutaneous neurofibromas were available for 44 of the patients. Counts of neurofibromas ranged from none to several hundred and appeared to increase logarithmically with the number of affected segments. I log-transformed the counts of neurofibromas and plotted them against number of divisions with 1 or more cutaneous neurofibromas to test whether the number of body segments with 1 or more cutaneous neurofibromas provided a good representation of the total number of cutaneous neurofibromas in an individual. I used linear regression to quantify this relationship (SPSS 1998). Counts of total number of café-au-lait spots were not made, and few subjects had more than one plexiform neurofibroma, so these variables were not analysed in this manner.

6.4.5 Analysis of familial factors

For the familial analysis, I stratified subjects into 5-year age intervals, calculated the deciles for the total number of segments affected with cutaneous neurofibromas in each stratum, and then ranked each subject by decile for the stratum in which he or she

lay. I then used random effects models to obtain maximum likelihood estimates and confidence intervals for intrafamilial correlations for decile (Spjotvoll 1967; Donner et al. 1989). Café-au-lait spots and plexiform neurofibromas were also analysed in the same manner.

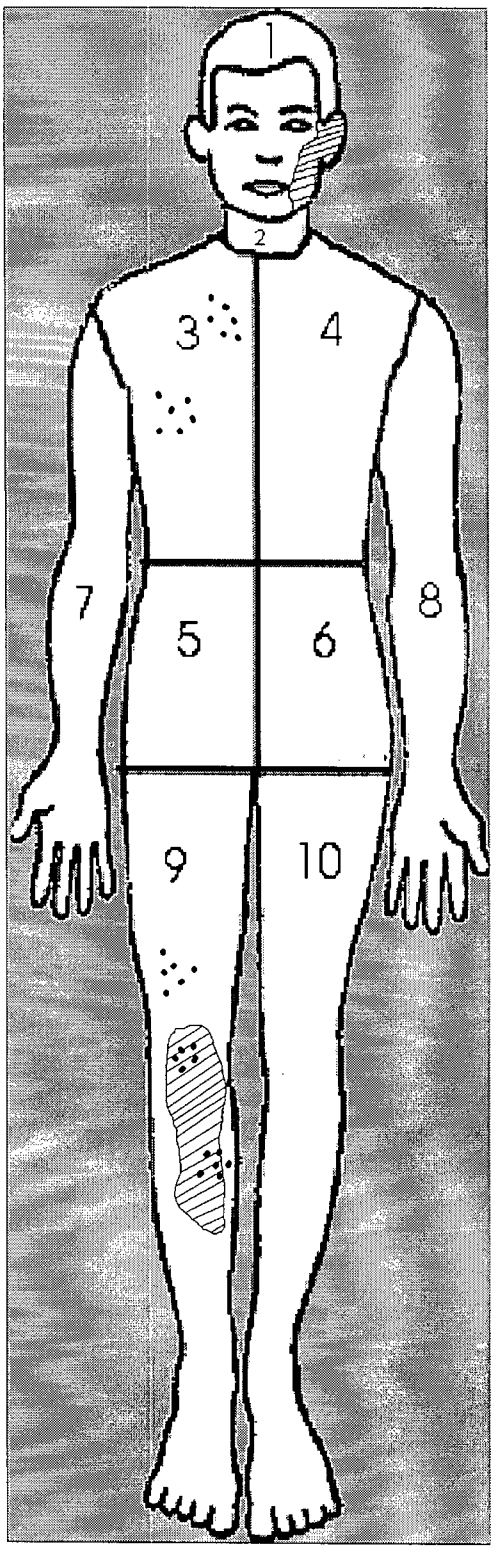
6.5 Results

I studied the recorded distributions of café-au-lait spots, cutaneous neurofibromas, and diffuse plexiform neurofibromas in 10 segments of the body surface of each of 547 patients with NF1. Figure 6.1 shows a front view of the 10 segments. Each segment also extends to the dorsal side of the body. Fifty-three percent (n=385) of the subjects were female, and 47% (n=344) male; 77% (n=426) were White, 12% (n=) were Hispanic, 8% (n=44) were Black and 1% (n=8) were of other or mixed origin. Mean age was 17 years, and median age was 12 years.

6.5.1 Lesion frequency by body segment

210 patients had no cutaneous neurofibromas in any segment. 4 patients had no café-au-lait spots in any segment. 331 patients had no plexiform neurofibromas in any segment. Table 6.1 shows the frequency of these lesions in each of the 10 body segments among the NF1 patients included in this study. Cutaneous and plexiform neurofibromas occurred with similar frequencies in all 10 body segments. Café-au-lait spots occurred in almost all patients in all body segments except the head and neck, where they were less frequent.

Figure 6.1: Body segment scheme used for recording location of lesions in the Neurofibromatosis Institute Database
Each of the 10 segments also extends to the dorsal side of the body.


1
2
3
4
5
6
7
8
9
10

6.5.2 Occurrence of various lesions in body segments is independent

Table 6.2 shows the 10 body segments examined and the pair-wise odds-ratio between neurofibromatosis lesions for each segment. No association was observed between the occurrence of cutaneous and diffuse plexiform neurofibromas in the same body segment. The summary odds-ratio was 1.20 (95% CI=0.81, 1.79). There was no evidence for heterogeneity across body divisions (p=0.37). Similarly, there was no association between the presence of café-au-lait spots and either cutaneous or diffuse plexiform neurofibromas within a single body segment. The summary odds-ratios were 1.36 (95% CI=0.91, 2.03) for café-au-lait spots and cutaneous neurofibromas and 1.25 (95% CI=0.74, 2.12) for café-au-lait spots and plexiform neurofibromas. There was no evidence of heterogeneity across body divisions for the occurrence of plexiform neurofibromas with café-au-lait spots (p=0.52), but there was significant (p=0.03) heterogeneity in the occurrence of cutaneous neurofibromas and café-au-lait spots, with a positive association seen in the neck (odds ratio=2.59; 95% CI=1.23, 5.47).

6.5.3 Relationship between segment size and number of neurofibromas

I observed no association between the relative size of the body surface area in a segment and the presence of one or more cutaneous neurofibromas (p=0.74) or of a diffuse plexiform neurofibroma (p=0.17). The number of body segments affected with one or more cutaneous neurofibromas was strongly correlated with the total number of cutaneous neurofibromas in 44 NF1 patients in whom both total counts and data on the number of affected body segments were available (r=0.95, p<0.001). The relationship is

Table 6.1: Distribution of lesions by body segment.
Number and percentage of patients with cutaneous neurofibromas, diffuse plexiform neurofibromas and café-au-lait spots by body segment in 547 NF1 patients.

		Cutaneous Neurofibromas		Plexiform Neurofibromas		Café-au-lait spots	
Segment		**Total**	**(%)**	**Total**	**(%)**	**Total**	**(%)**
1	Head	179	(33%)	47	(8%)	101	(18%)
2	Neck	168	(31%)	29	(5%)	397	(73%)
3	Right Upper Torso	259	(47%)	32	(6%)	532	(97%)
4	Left Upper Torso	258	(47%)	21	(4%)	531	(97%)
5	Right Lower Torso	285	(52%)	55	(10%)	537	(98%)
6	Left Lower Torso	287	(52%)	41	(7%)	533	(97%)
7	Right Arm	206	(38%)	21	(4%)	514	(94%)
8	Left Arm	208	(38%)	19	(3%)	511	(93%)
9	Right Leg	219	(40%)	54	(10%)	527	(96%)
10	Left Leg	220	(40%)	45	(8%)	525	(96%)
Total		***337***	***(62%)***	***216***	***(39%)***	***543***	***(99%)***

Table 6.2: Associations between lesions by body segment.
Associations between cutaneous neurofibromas, diffuse plexiform neurofibromas and café-au-lait spots by body segment in 547 NF1 patients.

		Cutaneous NFs vs. Plexiform NFs		Cutaneous NFs vs. Café-au-lait spots		Café-au-lait spots vs. Plexiform NFs	
Segment		**Odds Ratio**	**(95% C.I.)**	**Odds Ratio**	**(95% C.I.)**	**Odds Ratio**	**(95% C.I.)**
1	Head	0.95	(0.34-2.68)	1.34	(0.67-2.67)	1.26	(0.60-2.65)
2	Neck	2.39	(0.51-11.20)	2.59	(1.23-5.47)	2.42	(0.71-8.24)
3	Right Upper Torso	0.83	(0.23-3.02)	0.26	(0.01-10.34)	-	-
4	Left Upper Torso	0.39	(0.06-2.49)	0.12	(0.01-7.07)	1.29	(0.02-83.37)
5	Right Lower Torso	0.85	(0.32-2.24)	0.98	(0.01-84.41)	-	-
6	Left Lower Torso	0.91	(0.35-2.36)	1.13	(0.19-6.94)	0.06	(0.01-0.99)
7	Right Arm	1.17	(0.09-14.43)	1.91	(0.29-12.67)	-	-
8	Left Arm	1.01	(0.18-5.60)	0.91	(0.18-4.65)	0.22	(0.02-1.97)
9	Right Leg	3.91	(1.02-15.06)	0.20	(0.04-1.15)	0.35	(0.05-2.34)
10	Left Leg	3.60	(0.99-13.08)	1.10	(0.24-5.00)	2.70	(0.17-44.12)
Summary		***1.20***	***(0.81-1.79)***	***1.36***	***(0.91-2.03)***	***1.25***	***(0.74-2.12)***

log linear (Figure 6.2); the regression equation is log(total number of neurofibromas +1) = 0.23*(number of segments affected) + 0.014.

6.5.4 Familial correlations

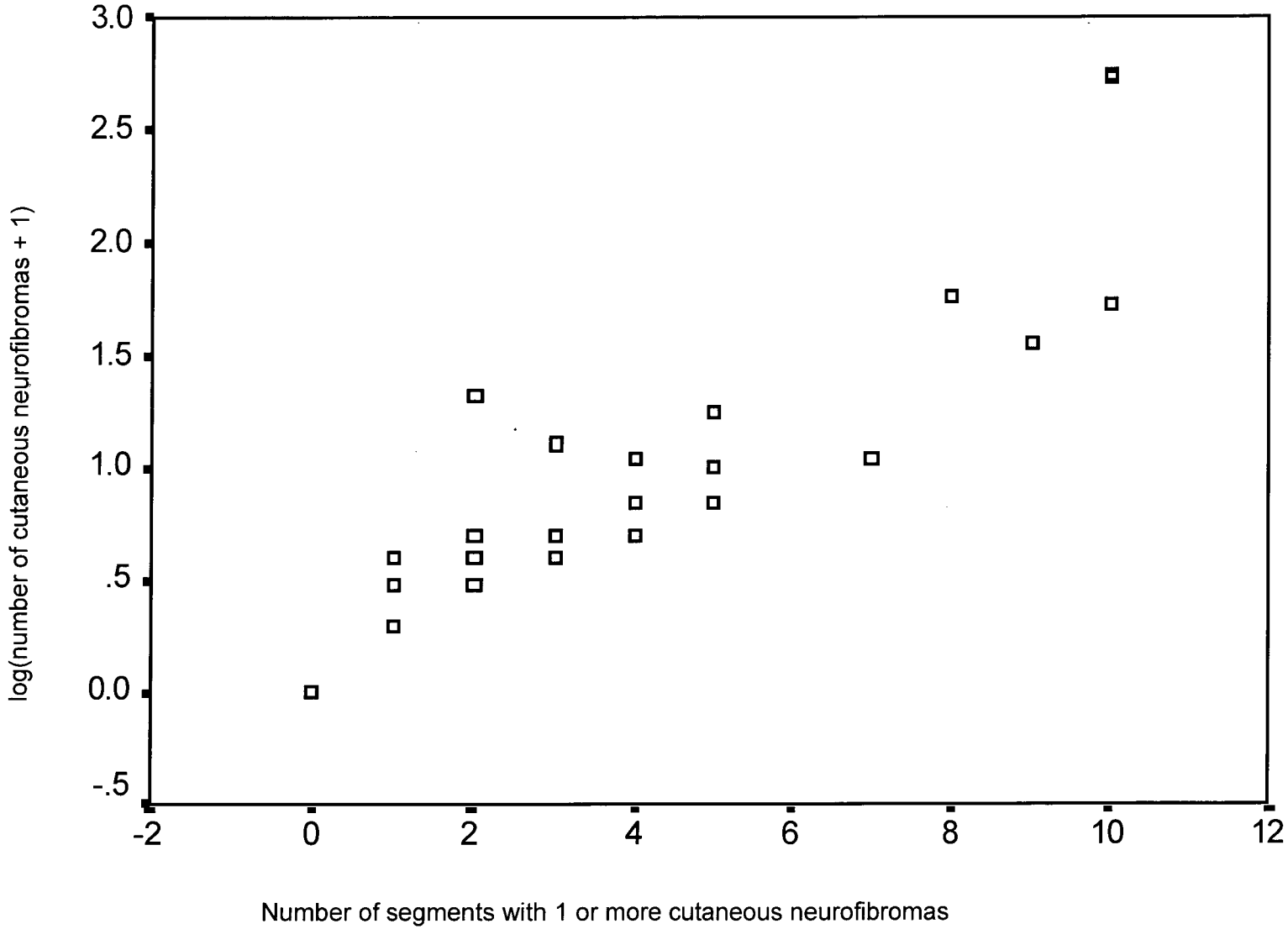
I estimated intrafamilial correlations in the age-adjusted number of body segments (age-specific decile for number of affected segments) that included one or more cutaneous neurofibromas, one or more café-au-lait spots, or one or more plexiform neurofibromas in 117 affected members of 52 families. I found significant intrafamilial correlations in the number of body segments affected by each of these clinical features. The correlation among relatives in the age-adjusted number of body segments with 1 or more cutaneous neurofibromas was 0.37 (95% CI=0.15,0.55). The correlation among relatives in the age-adjusted number of body segments with 1 or more plexiform neurofibromas was 0.35 (95% CI=0.15,0.57). The correlation among relatives in the age-adjusted number of body segments with 1 or more café-au-lait spots was 0.45 (95% CI=0.18,0.71).

6.6 Discussion

6.6.1 Lesion severity

The number of body segments affected by 1 or more cutaneous neurofibromas appears to provide a good measure of how severely each of these NF1 patients was affected by this disease feature. I found a very high correlation with the number of body segments affected in 44 patients for whom counts of the total number of cutaneous

Figure 6.2: Number of affected segments versus number of neurofibromas. Correlation between the number of body segments affected with one or more cutaneous neurofibromas and the total number of cutaneous neurofibromas in 44 NF1 patients ($r=0.95$, $p<0.001$). Total neurofibroma counts and data on the number of affected body segments were available in all patients. The relationship is log linear; the regression equation is Log(total number of neurofibromas +1) = 0.23*(number of segments affected) + 0.014.


3.0
2.5
2.0
1.5
1.0
.5
0.0
-.5
log(number of cutaneous neurofibromas + 1)
-2
0
2
4
6
8
10
12
Number of segments with 1 or more cutaneous neurofibromas

neurofibromas were available (Figure 6.2). It seems likely that a correlation also exists between the number of body segments affected with café-au-lait spots or plexiform neurofibromas and the severity of each of these disease features, but count data were not available in this study to demonstrate this.

6.6.2 Ascertainment issues

The subjects were referred to a specialised clinic, so I was concerned that they are more severely affected than the NF1 patient population at large. The frequencies of cutaneous neurofibromas, plexiform neurofibromas and café-au-lait spots are comparable to those from another large database (Friedman and Birch 1997b) and population based studies (Samuelsson and Axelsson 1981; Huson et al. 1989a; McGaughran et al. 1999). All of the subjects in this study were examined by the same clinician, ruling out the bias inherent in using multiple examiners. Body segment data were collected over a single visit for each patient, but the collection period lasted 16 years. I expect this to make the data less consistent than if they were gathered over a shorter period of time.

6.6.3 Local associations of lesions

I have shown in Chapter 2 that individuals with diffuse plexiform neurofibromas are more likely also to have cutaneous neurofibromas, but this association did not take into account the location or number of these lesions. The current study is the first to examine this association within body divisions. Since virtually all diffuse plexiform neurofibromas are of congenital origin (Riccardi 1992), I wanted to find out if they

influence the subsequent development of cutaneous neurofibromas. These findings suggest that the occurrence of cutaneous neurofibromas in NF1 patients is not influenced by the local presence of a diffuse plexiform neurofibroma. In fact, I found that all three of the lesions studied (café-au-lait spots, cutaneous neurofibromas, and plexiform neurofibromas) occurred independently of each another in almost all of the body segments analysed (Table 6.2). This is consistent with an independent triggering mechanism for each lesion.

6.6.4 Local factors in pathogenesis

Mast cells have been implicated in the pathogenesis of plexiform neurofibromas (Giorno et al. 1989). It has been hypothesised that histamine or other products secreted by these cells may influence the growth of neurofibromas, either the plexiform neurofibroma itself or other cutaneous or subcutaneous lesions (Riccardi 1992). This effect would presumably be strongest near the original plexiform neurofibroma. My results argue against the hypothesis that factors secreted by a diffuse plexiform neurofibroma stimulate the development of nearby cutaneous neurofibromas.

Local trauma has also been implicated in the pathogenesis of neurofibromatosis lesions. Cutaneous neurofibromas may arise in an area that has been injured (Riccardi 1990). Freckles and other areas of hyperpigmentation tend to occur in skin folds, presumably due to local environmental factors (Fitzpatrick 1981; Riccardi 1992). I found a significant association between café-au-lait spots and cutaneous neurofibromas only in the neck segment. Café-au-lait spots were found on all segments except the head and neck in almost all patients. Most body segments may lack the variation needed to

observe an association, although a strong (but not statistically significant) association was observed between café-au-lait spots and plexiform neurofibromas on the left leg (Table 6.2). The result could also be due to chance alone, since many different associations were calculated. A pathological reason the neck might be affected by both lesions is recurrent minor trauma to the skin associated with flexion, extension, and rotation of the head (Riccardi 1990). It would be interesting to study local associations in more detail by checking specific lesions, such as cutaneous neurofibromas, tend to aggregate in the same body segment. Clearly, however, other factors are also involved in the pathogenesis of café-au-lait spots and neurofibromas, as indicated by the familial correlations I observed for the age-adjusted number of body segments affected by each of the three lesions studied.

6.6.5 Familial associations

The significant intrafamilial correlations I found are consistent with other evidence that familial factors contribute to the development of cutaneous neurofibromas and café-au-lait spots in patients with NF1 (Easton et al. 1993) (see Chapters 2 and 7). The number of familial patients and the prevalences of cutaneous and plexiform neurofibromas and café-au-lait spots in patients were similar among the three study groups (Riccardi 1992; Easton et al. 1993; Friedman and Birch 1997b). The present study found a similar correlation for café-au-lait spots but higher correlations for cutaneous neurofibromas than Easton et al.. I also found a significant correlation for plexiform neurofibromas, whereas Easton only analysed this feature as a binary trait and found no familial association. My use of 10 body segments allows for more precise

stratification than Easton's 5 ordinal categories and may account partly for the differences.

6.6.6 Familial factors in pathogenesis

These familial associations suggest a genetic influence on the severity of NF1 lesions. Contributing factors may include effects of the mutant *NF1* allele itself, effects of the normal *NF1* allele, or modifying effects of other loci. The moderate magnitudes of the intrafamilial correlation coefficients show that family history alone is insufficient to predict the degree to which a patient will be affected with these lesions. Other elements must also be involved.

Previous studies support the hypothesis that some patients are predisposed to lesions such as café-au-lait spots, cutaneous neurofibromas and plexiform neurofibromas. The results of this chapter are consistent with the possibility that different pathogenetic mechanisms are responsible for the three lesions studied. Cutaneous neurofibromas are composed of Schwann cells, fibroblasts, mast cells and nerve cell axons (Korf 1999a). Schwann cells are thought to play a major role in tumourgenesis since they have been found to undergo loss of heterozygosity at the *NF1* locus, while other neurofibroma cells have not (Kluwe et al. 1999; Rutkowski et al. 2000). Although the mechanism by which loss of *NF1* function leads to neurofibromas is unknown, Schwann cells migrate during embryonic development from the neural crest (Johnston et al. 1981) and give rise to cutaneous neurofibromas arise through neoplasia (excess cellular division), at least in many cases (Stark et al. 1995).

Although plexiform neurofibromas also contain Schwann cells, fibroblasts, mast

cells and axons, they are congenital and have more characteristics of dysplasia (irregular tissue arrangement) than do cutaneous neurofibromas (Riccardi 1992). Plexiform neurofibromas usually have extensive vascularisation, involve many different tissues and can spread to distort adjacent tissues (Korf 1999a).

Chimeric mice composed in part of *Nf1*$^{-/-}$ cells develop plexiform neurofibromas but not cutaneous neurofibromas (Cichowski et al. 1999; Vogel et al. 1999). On the other hand, transgenic mice expressing the human T-lymphotropic virus type 1 *tax* gene develop cutaneous neurofibromas (Hinrichs et al. 1987; Nerenberg et al. 1987; Green et al. 1992; Feigenbaum et al. 1996). Although repression of *Nf1* expression by *tax* occurs in the absence of mutation at the *Nf1* locus, these observations suggests that plexiform and discrete neurofibromas can arise by pathways that are independent, at least in mice.

Like neurofibromas, café-au-lait spots contain neural crest-derived cells, but these are melanocytes with abnormally large pigment particles (Fitzpatrick 1981) rather than Schwann cells, as in neurofibromas. Some families with *NF1* mutations develop café-au-lait spots but no tumours, consistent with different pathogenetic factors being involved in the development of neurofibromas (Abeliovich et al. 1995).

6.7 Conclusion

These findings are consistent with multiple factors being involved in the pathogenesis of both plexiform and cutaneous neurofibromas as well as of café-au-lait spots. Some of these factors appear to be genetic, but others do not. Although some of the pathogenetic factors may be shared among these three lesions, others appear to differ.

7. ANALYSIS OF INTRA-FAMILIAL PHENOTYPIC VARIATION IN NEUROFIBROMATOSIS 1

7.1 Hypothesis

Genetic sources of variable expressivity are generally important in NF1 and vary for different clinical features.

7.2 Objective

To measure associations of NF1 features among affected sibs, children and their mothers and fathers, and 2nd degree relatives using methods that take other clinical features and age into account and adjust for the non-independence of affected relatives.

7.3 Introduction

Many clinical features of NF1 are progressive, but the rate of progression and the occurrence of serious manifestations vary greatly from one patient to another (Friedman and Riccardi 1999). This variability and the confounding effect of age have hindered efforts to characterise the relationship of genetic factors at the *NF1* locus or other loci to disease variability.

The large size of the *NF1* gene makes mutational analysis difficult and there is not much evidence for allele-phenotype correlations in most NF1 patients (see section 1.13). Variation in the mutant *NF1* allele itself is insufficient to account for the variability of most disease features. Mouse models provide evidence that genetic factors at other loci can affect the phenotype associated with *Nf1* mutations but are limited to only a few of NF1 disease features observed in humans (see section 1.11). Easton et al. (1993) found evidence for genetic factors influencing the number or presence of several features in

NF1 patients. These results rely heavily on near-perfect concordance among 6 pairs of monozygotic twins and some were not adjusted for the non-independence of multiple relative-pairs from the same family.

I have shown in Chapters 2 and 4 that several statistically significant associations exist between the occurrence of individual clinical features in unrelated probands with NF1. The results in Chapter 6 suggest that café-au-lait spots, cutaneous neurofibromas and plexiform neurofibromas are influenced by familial factors. I also found significant associations in the occurrence of Lisch nodules, optic glioma, learning disability, macrocephaly and short stature in affected parent-child pairs (see Chapter 2) but made no attempt to adjust for the non-independence of multiple relative-pairs from the same family or for associations among clinical features in individuals in that preliminary study.

The statistical methodology for testing for such non-independence is well developed for continuous variables and is widely available as part of software packages such as S.A.G.E. (1997). Many NF1 clinical features are discrete by nature, and the methodology for this kind of analysis is not well developed for discrete variables. Prior to this study, the only available programme was Generalized Estimating Equations 2 (GEE2), but it did not allow for complete intra-familial relationship classifications (Liang and Beaty 1991). In this study, I used newly revised programmes for multivariate probit analysis (MPROBIT) and multivariate normal analysis (MVNFAM) to extend my previous analyses (Joe 2000). I measured associations of NF1 features among affected sibs, children and their mothers and fathers, and 2nd degree relatives using methods that take other clinical features and age into account and adjust for the non-independence of affected relatives. By comparing the associations for relatives of various types, I provide

evidence that genetic sources of variable expressivity are generally important in NF1 and vary for different clinical features.

7.4 Subjects and methods

7.4.1 Subjects

All patients in this study meet the NIH diagnostic criteria for NF1 (NIH 1988; Gutmann et al. 1997). Data were obtained from the National NF Foundation International Database (NFDB) (Friedman et al. 1993) on 913 individuals from 373 families with 2 or more affected members, including 268 sib-sib, 373 parent-child and 74 2nd degree relative pairs.

7.4.2 Features

For analysis of familiality, I selected 12 clinical features of NF1: café-au-lait spots, intertriginous freckling, Lisch nodules, cutaneous neurofibromas, subcutaneous neurofibromas, plexiform neurofibromas, head circumference, stature, seizures, scoliosis, optic glioma and neoplasms other than neurofibromas and optic gliomas ("other neoplasms"). Most of the features were identified by physical examination and treated as binary variables. The NIH Criteria (NIH 1988) (Table 1.1) were used whenever applicable. Café-au-lait spots were coded as "present" if the subject had 6 or more spots. Cutaneous or subcutaneous neurofibromas were coded as "present" if the subject had two or more lesions of the same type. Plexiform neurofibroma was coded as "present" if the subject had one or more lesions. Stature and head circumference were treated as

continuous variables and standardised prior to analysis using population norms (see section 3.4.3). Lisch nodules were diagnosed or excluded by a slit lamp examination. The presence or absence of optic glioma was determined by cranial MRI or CT examination. Only patients with definite presence or absence of a feature were considered in models involving that feature. The complete data set used in this study is available from ftp://ftp.stat.ubc.ca/pub/hjoe.

7.4.3 Confounding factors

I included age as a covariate in all analyses. Age is one of the most important factors influencing the NF1 phenotype (Zöller et al. 1995). Many NF1 features, including Lisch nodules, subcutaneous neurofibromas, cutaneous neurofibromas, other neoplasms, intertriginous freckling, seizures and scoliosis have a higher prevalence in older patients. This is illustrated by DeBella et al. (2000b) and in Figures 4.1-4.13. Furthermore, clinical features do not occur independently in NF1 patients, even after adjusting for the effect of age (see Chapters 2 and 4). Therefore, I also controlled for the presence or absence of other associated features to minimise confounding in the present study.

7.4.4 Multivariate probit and normal models

My general approach was to treat the features of NF1 as if each were a disorder occurring in a population affected with NF1. Familial aggregation of each binary feature among various classes of relatives was estimated using multivariate probit regression

models (Joe 1997), which assume that each of the binary dependent features reflects an underlying latent quantitative variable (Thompson et al. 1991). Whereas linear regression attempts to compute the mathematical relationship between the covariates and the response variable directly, probit regression attempts to compute the relationship between the covariates and the probability of the response variable being "present" – expressed in terms of the normal distribution. Familial aggregation of continuous features (head circumference and stature) among various classes of relatives was estimated using multivariate normal regression models. This is similar to probit regression except that the continuous variable is modelled directly, rather than as a probability. The program MPROBIT was used for binary features and MVNFAM for continuous features (Joe 2000).

Multivariate probit regression consists of two sets of parameters: regression parameters for covariates such as related features, interactions between related features (each represented by a distinct variable equal to the product of the two interacting features), age and gender; latent correlation parameters for binary features between specific classes of relatives. For head circumference and stature, the second set of parameters are correlations (not latent correlations) of the continuous feature between specific classes of relatives. MPROBIT and MVNFAM provide maximum likelihood estimates of the regression coefficients together with standard errors. The programmes estimate the correlation or latent correlation coefficients and standard errors for the intra-familial relationships specified and a covariance matrix of all parameter estimates.

I used the results of my study of individuals with NF1 (see Chapter 4) to obtain appropriate functions for age (e.g. $e^{-age/4}$) and initial regression parameter estimates for

covariates representing related features, interactions between related features and gender. Familial aggregation was assessed among sibs, parent-child pairs (including mother-child and father-child pairs separately) and 2nd degree relatives.

7.4.5 Assessment of intrafamilial correlations

Parameters and coefficients with 95% confidence intervals that excluded zero were deemed statistically significant. Standard errors and covariance matrices were used to test for differences between intra-familial correlation coefficients for different comparisons. For example, to test for a difference between sib-sib correlation and parent-child correlation I used the following formulas:

$$Z = \frac{r_{ss} - r_{pc}}{s} \quad \text{where} \quad s = \sqrt{(SE_{rss})^2 + (SE_{rpc})^2 - 2\,\mathrm{cov}(r_{ss}, r_{pc})}$$

Z-scores were converted into p-values according to the standard normal distribution. I used one-tailed tests to compare correlations between 1st degree and 2nd degree relatives and between sib pairs and parent-child pairs because I had a prior expectation that correlations between 1st degree relatives would be at least as strong as those between 2nd degree relatives (Easton et al. 1993) and that correlations between sibs would be at least as strong as those between parents and children (see Chapter 2). I used two-tailed tests to compare mother-child correlations to father-child correlations.

7.5 Results

7.5.1 Feature prevalences

I studied 913 individuals with NF1 from 373 families with two or more affected members. 91% of the individuals studied were White, 2% Asian, 1% Black, 1% Latin, and the remainder either of "other" or "unknown" origin. Table 7.1 shows the prevalences of each of the 12 NF1 clinical features in affected fathers, mothers and their affected children in this NFDB study sample and compares them to the prevalences in the sample used by Easton et al. (1993).

7.5.2 Individual covariates

Familial aggregation among various classes of relatives was estimated using multivariate probit and multivariate normal regression models. Table 7.2 shows the regression parameters and standard errors for the covariates that were included in each model. The importance of these covariates was first identified in Chapter 4. Unlike the parameter estimates in Table 4.2, the estimates in Table 7.2 have been calculated for each feature taking into account intra-familial associations (see next section). The strength of association between the modelled feature and a covariate is measured by β. The regression coefficient in probit regression is approximately half that for logistic regression. A unit increase in the value of the covariate means the modelled feature is $\exp(2\beta)$ times more likely to be present. For example, subjects with intertriginous freckling were $\exp(2\times.51)=2.8$ times more likely also to have café-au-lait spots than

Table 7.1: Comparison of NF1 feature frequencies from two studies.
Number and percentage of subjects from the NFDB and from the study by Easton et al. (1993) with various NF1 features. Features not considered by Easton et al. have empty cells in the last column.

	NFDB								Easton et al. (1993)	
Feature	**Affected Fathers**		**Affected Mothers**		**Children of Affected Fathers**		**Children of Affected Mothers**		**All Affected Relatives**		**All Affected Relatives**	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Lisch nodules	63	(81%)	101	(80%)	64	(63%)	101	(51%)	409	(60%)		
Café-au-lait macules	67	(68%)	125	(73%)	115	(79%)	181	(74%)	657	(75%)	129	(88%)
Cutaneous neurofibromas	78	(80%)	119	(69%)	37	(25%)	67	(27%)	355	(40%)	121	(76%)
Optic glioma	4	(13%)	2	(4%)	8	(14%)	14	(15%)	45	(15%)	9	(5%)
Subcutaneous neurofibromas	60	(61%)	99	(59%)	31	(21%)	58	(23%)	291	(33%)		
Intertriginous freckling	79	(83%)	147	(88%)	112	(78%)	191	(78%)	699	(80%)		
Seizures	7	(7%)	12	(7%)	8	(5%)	16	(6%)	58	(6%)	12	(7%)
Plexiform neurofibromas	24	(24%)	35	(20%)	25	(18%)	44	(18%)	176	(20%)	37	(21%)
Scoliosis	7	(8%)	8	(6%)	29	(22%)	32	(14%)	96	(12%)	27	(16%)
Other neoplasms	4	(4%)	11	(6%)	4	(3%)	7	(3%)	33	(4%)		

Table 7.2: Summary of multivariate normal and probit models of NF1 clinical features.

Summary of regressions in multivariate models for 12 clinical NF1 features. The 1st column lists the 12 modelled features. The 2nd–4th columns show the covariates and their regression parameter estimates (β) with standard errors (SE) used in each model. β_0 is the intercept in the model equation. Each regression accounts for covariates such as related features, interactions between related features, age and gender. Interactions are depicted by features separated by an "*" and their values equal the product of the two interacting features.

Modelled Feature	Intercept and Covariates	β	SE
Lisch nodules	β_0	.65	(.08)
	Age	-3.55	(.32)
	Male gender	-.01	(.08)
	CLS	.23	(.15)
	CNF	.44	(.20)
	CLS * CNF	-.09	(.22)
Café-au-lait spots (CLS)	β_0	.28	(.14)
	Age	-.66	(.25)
	Male gender	.03	(.09)
	Freckling	.51	(.12)
	SNF	-.41	(.26)
	Freckling * SNF	.61	(.28)
Head circumference (OFC)	β_0	-.99	(.10)
	Age	.62	(.21)
	Male gender	-.09	(.31)
	Lisch nodules	-.06	(.36)
	Optic glioma	.56	(.44)
	Stature	.34	(.04)
	Neoplasms	.10	(.75)
Cutaneous neurofibromas (CNF)	β_0	-1.62	(.11)
	Age	-5.56	(.36)
	Male gender	.01	(.10)
	SNF	.62	(.11)
	PNF	.36	(.12)
Stature	β_0	-.62	(.09)
	Age	-.82	(.31)
	Male gender	-.03	(.09)
	OFC	.04	(.01)
Optic glioma	β_0	-1.02	(.13)
	Age	.72	(.57)
	Male gender	.06	(.17)
	PNF	.01	(.37)
	OFC	.19	(.07)
	Neoplasms	.55	(.49)

Table 7.2 continued:

Modelled Feature	Intercept and Covariates	β	SE
Subcutaneous neurofibromas (SNF)	β_0	-1.72	(.12)
	Age	-3.78	(.35)
	Male gender	-.04	(.08)
	CLS	.43	(.11)
	CNF	.73	(.13)
	PNF	.52	(.17)
	Freckling * PNF	-.24	(.23)
Intertriginous freckling (Freckling)	β_0	.49	(.15)
	Age	-1.58	(.30)
	Male gender	-.23	(.12)
	CLS	.52	(.14)
	SNF	-.18	(.27)
	Lisch nodules	.55	(.14)
	CLS * SNF	.62	(.33)
Seizures	β_0	-1.43	(.11)
	Age	-.88	(.65)
	Male gender	-.04	(.15)
Plexiform neurofibromas (PNF)	β_0	-1.11	(.11)
	Age	-.88	(.38)
	Male gender	.07	(.09)
	SNF	.46	(.16)
	CNF	.37	(.14)
	SNF * CNF	-.21	(.22)
Scoliosis	β_0	-1.11	(.09)
	Age	-.57	(.34)
	Male gender	-.02	(.11)
Other neoplasms	β_0	-.95	(.23)
	Age	-4.07	(2.11)
	Male gender	-.06	(.21)
	Lisch nodules	-.55	(.25)
	Optic glioma	.32	(.31)

subjects of the same age and gender without intertriginous freckling. Also, subjects with intertriginous freckling *and* subcutaneous neurofibromas were exp [2×(.51-.41+.61)]=4.1 times more likely to also have café-au-lait spots.

The parameter estimates for age were highly significant ($p<0.001$) for Lisch nodules, subcutaneous neurofibromas, cutaneous neurofibromas, and intertriginous freckling; significant ($p<0.05$) for café-au-lait spots, optic gliomas, plexiform neurofibromas, and the continuous variables head circumference and stature; and not significant ($p>0.05$) for other neoplasms, seizures and scoliosis. The parameter estimate for gender was not significant in any of the models. However, parameters that are not statistically significant on their own can still contribute to model interpretation and significance when other related features are also considered.

7.5.3 Intrafamilial associations

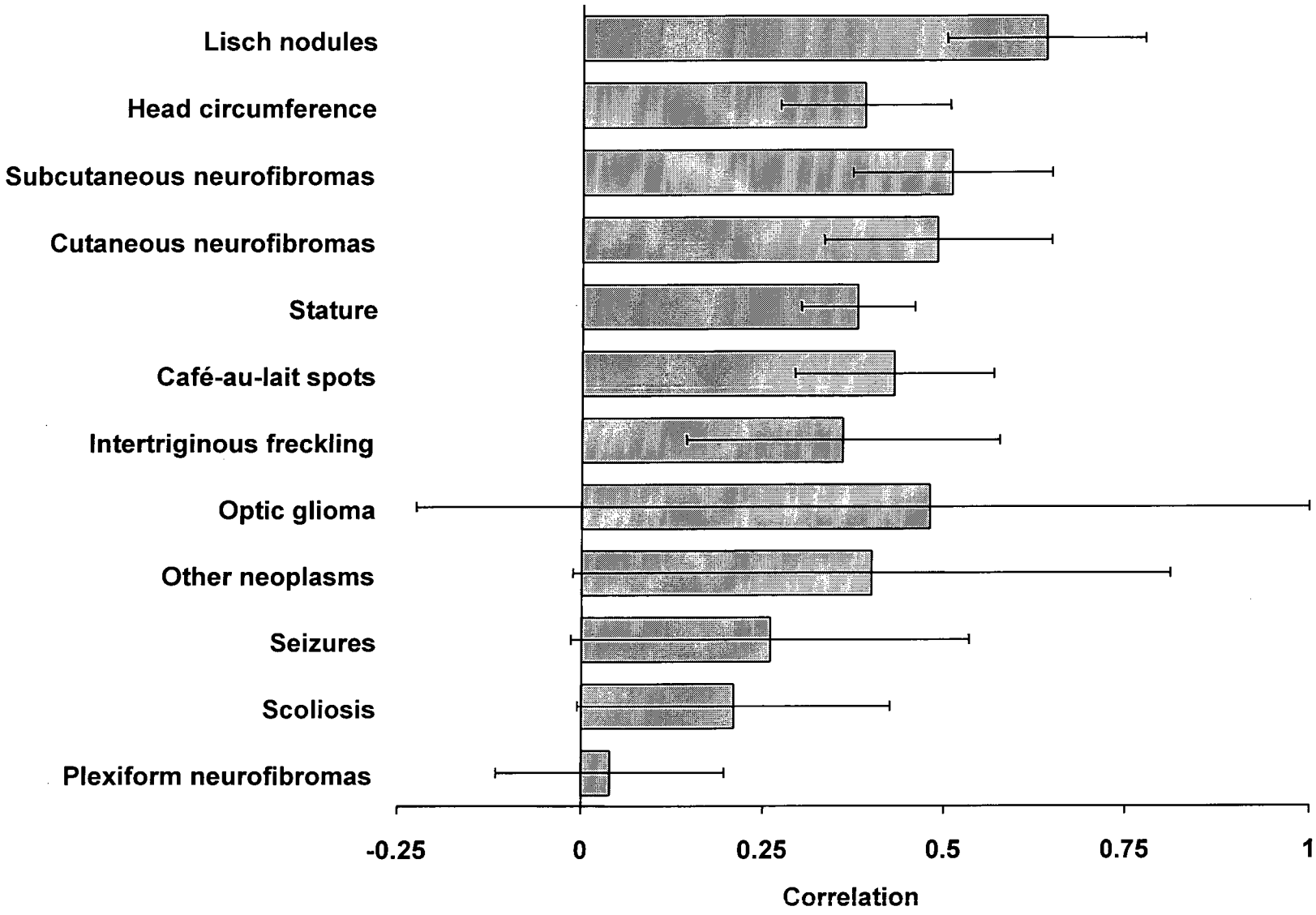
Table 7.3 shows the number of sib, parent-child (including mother-child and father child) and 2nd degree relative pairs used in each model. Subjects were included in a model only if the status ("presence" or "absence") of the modelled feature and all covariates was known.

The measure of association for multivariate probit analysis of binary variables is the *latent correlation.* The measure of association for multivariate normal analysis of continuous variables is the *correlation.* Figure 7.1 shows the adjusted intrafamilial correlation or latent correlation coefficients and their 95% confidence intervals for each

Table 7.3: Number of relatives used in multivariate models of NF1 features.
The 1st column lists the 12 modelled features. The 2nd-5th columns show the number of affected sib, mother-child, father-child and 2nd degree relative pairs for which the status of the modelled feature and covariates in Table 7.2 is known.

Modelled Feature	Sib Pairs	Mother-Child Pairs	Father-Child Pairs	2° Relative Pairs
Lisch nodules	192	159	79	35
Café-au-lait macules	248	210	129	69
Head circumference	103	64	37	24
Cutaneous neurofibromas	264	224	131	69
Stature	103	64	37	24
Optic glioma	55	37	26	4
Subcutaneous neurofibromas	253	220	131	69
Intertriginous freckling	179	148	75	35
Seizures	268	233	140	74
Plexiform neurofibromas	264	224	131	69
Scoliosis	228	191	131	53
Other neoplasms	47	33	20	3

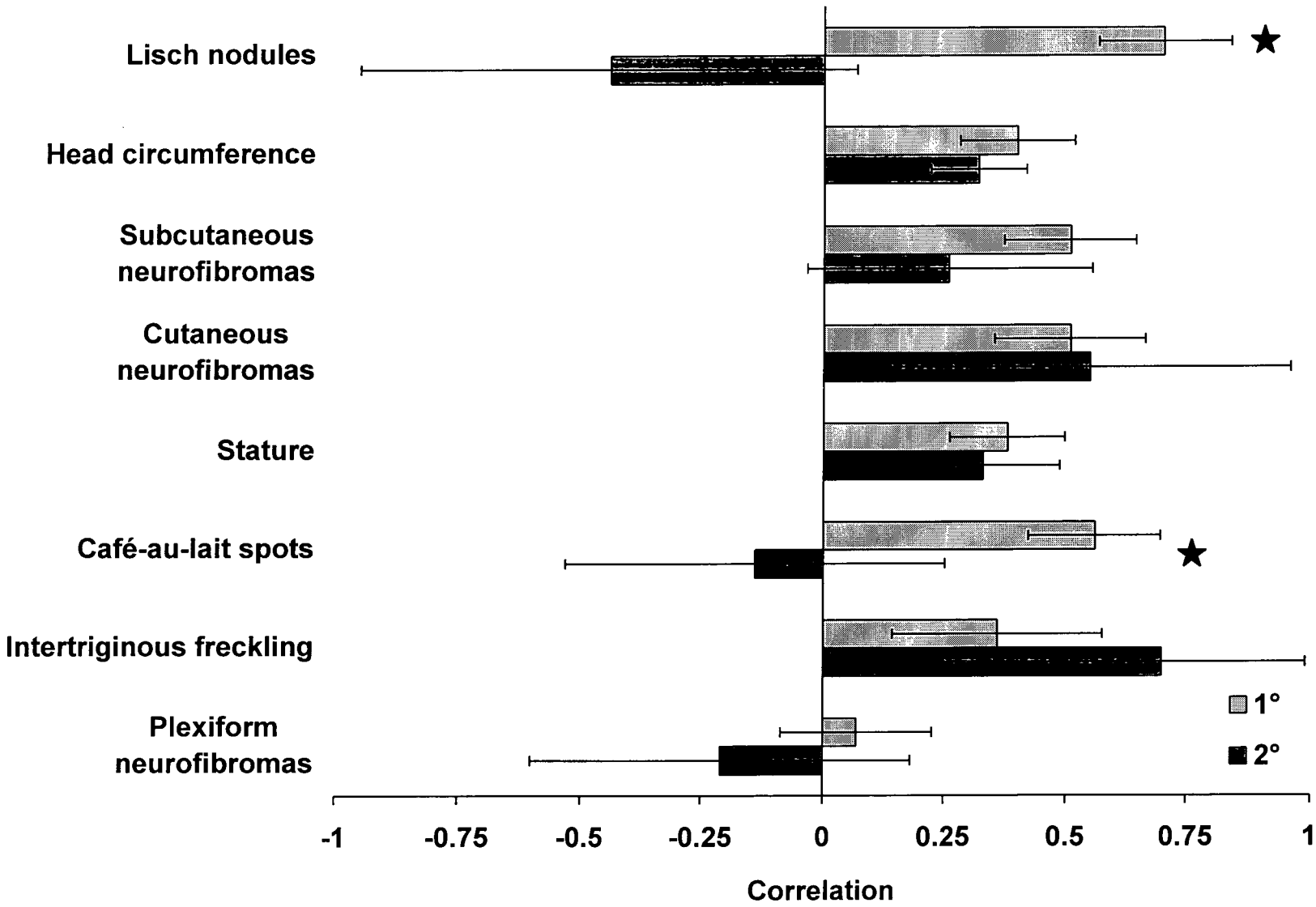
Figure 7.1: Feature associations among relatives with NF1.
Adjusted intrafamilial correlation or latent correlation coefficients and their 95% confidence intervals are shown for each of the 12 features among all 913 relatives with NF1 from the 373 families studied. In these estimates, all relatives are treated the same regardless of relationship.


Lisch nodules
Head circumference
Subcutaneous neurofibromas
Cutaneous neurofibromas
Stature
Café-au-lait spots
Intertriginous freckling
Optic glioma
Other neoplasms
Seizures
Scoliosis
Plexiform neurofibromas
-0.25
0
0.25
0.5
0.75
1
Correlation

of the 12 features among all 913 relatives with NF1 from the 373 families studied. In these estimates, all relatives are treated the same regardless of relationship. Statistically significant positive intrafamilial correlations or latent correlations were observed for Lisch nodules, head circumference, subcutaneous neurofibromas, cutaneous neurofibromas, stature, café-au-lait spots and intertriginous freckling. Latent correlations for optic glioma, other neoplasms, seizures, scoliosis and plexiform neurofibromas, although positive, were not statistically different from zero. However, the number of individuals who had the latter features, especially optic glioma, other neoplasms, or seizures, was small, and the confidence intervals are very wide.

Figure 7.2 shows the adjusted intrafamilial correlation or latent correlation coefficients and 95% confidence intervals for 8 clinical features among 746 affected 1st degree relatives and among 148 affected 2nd degree relatives. MPROBIT failed to converge on latent correlation coefficients between 2nd degree relatives for optic glioma, other neoplasms, seizures or scoliosis because of the low frequency of these features and insufficient sample size. I did obtain latent correlation coefficients between 1st degree relatives for these features, but none was significantly different from zero. Statistically significant positive correlations or latent correlations between 1st degree relatives were found for 7 of the 8 other features listed in Figure 7.1. Significant positive correlations or latent correlations between 2nd degree relatives were also found for 4 of these 8 features. Significant negative correlations were not observed for any of the features. Latent correlations were significantly greater among 1st degree relatives than among 2nd degree relatives for Lisch nodules ($p=0.0001$) and café-au-lait spots ($p=0.0004$). Correlations or

Figure 7.2: Feature associations among 1st and 2nd degree relatives with NF1. Adjusted intrafamilial correlation or latent correlation coefficients and 95% confidence intervals are shown for 8 clinical features among 746 affected 1st degree relatives and among 148 affected 2nd degree relatives. A star indicates a significant difference between the coefficient among 1st degree relatives and the coefficient among 2nd degree relatives. MPROBIT failed to converge on latent correlation coefficients among 2nd degree relatives for optic glioma, other neoplasms, seizures or scoliosis because of the low frequency of these features and insufficient sample size.


Lisch nodules
Head circumference
Subcutaneous neurofibromas
Cutaneous neurofibromas
Stature
Café-au-lait spots
Intertriginous freckling
Plexiform neurofibromas
1°
2°
-1
-0.75
-0.5
-0.25
0
0.25
0.5
0.75
1
Correlation

latent correlations among 1st degree relatives were not statistically different from correlations among 2nd degree relatives for head circumference (p=0.15), subcutaneous neurofibromas (p=0.06), cutaneous neurofibromas (p=0.49), stature (p=0.30), intertriginous freckling (p=0.07) or plexiform neurofibromas (p=0.11).

Figure 7.3 shows the adjusted intrafamilial correlation or latent correlation coefficients and 95% confidence intervals for 8 features among 268 affected sib pairs and among 373 affected parent-child pairs. Again, MPROBIT failed to converge on latent correlation coefficients between sib or parent-child pairs for optic glioma, other neoplasms, seizures or scoliosis. Statistically significant positive correlations or latent correlations between sibs were found for all 8 features in Figure 7.3. Significant positive correlations or latent correlations between parents and children were found for 6 of the 8 features. Significant negative correlations were not observed for any of the features. Latent correlations were significantly greater between sibs than between parents and children for subcutaneous neurofibromas (p=0.04), café-au-lait spots (p=0.001), intertriginous freckling (p=0.03) and plexiform neurofibromas (p=0.02). Correlations or latent correlations between sibs were not statistically different from the correlations between parents and children for Lisch nodules (p=0.40), head circumference (p=0.45), cutaneous neurofibromas (p=0.29), or stature (p=0.20).

Figure 7.4 shows the adjusted intrafamilial correlation or latent correlation coefficients and 95% confidence intervals for 8 features between 233 affected mother-child pairs and between 140 affected father-child pairs. Statistically significant positive correlations or latent correlations between mothers and children were found for 5 of the 8 features. Significant positive correlations or latent correlations between fathers and

Figure 7.3: Feature associations among sib and parent-child pairs with NF1. Adjusted intrafamilial correlation or latent correlation coefficients and 95% confidence intervals are shown for 8 features among 268 affected sib pairs and among 373 affected parent-child pairs. A star indicates a significant difference between the coefficient among sibs and the coefficient among parents and children. MPROBIT failed to converge on latent correlation coefficients among sibs or parent-child pairs for optic glioma, other neoplasms, seizures or scoliosis.

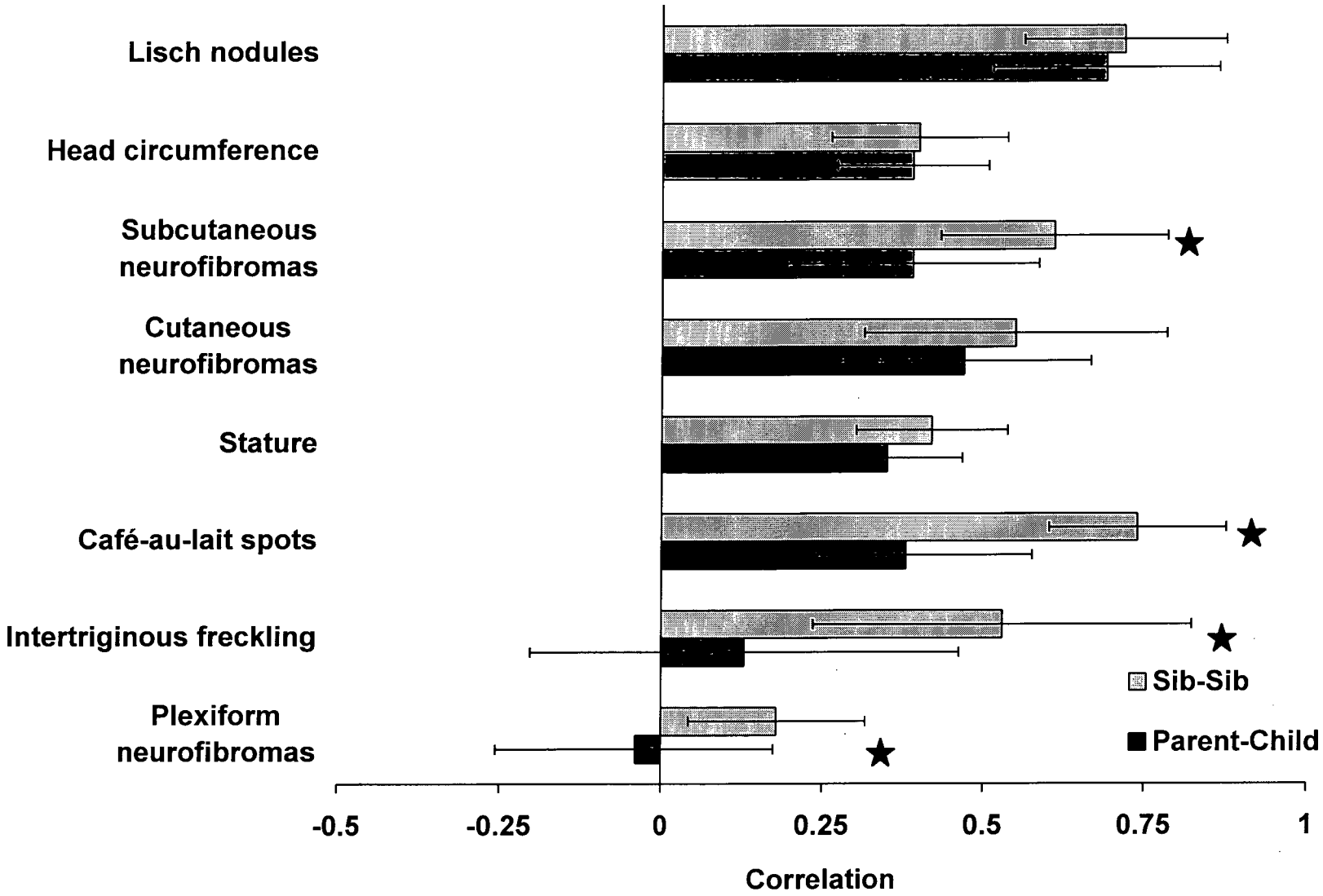
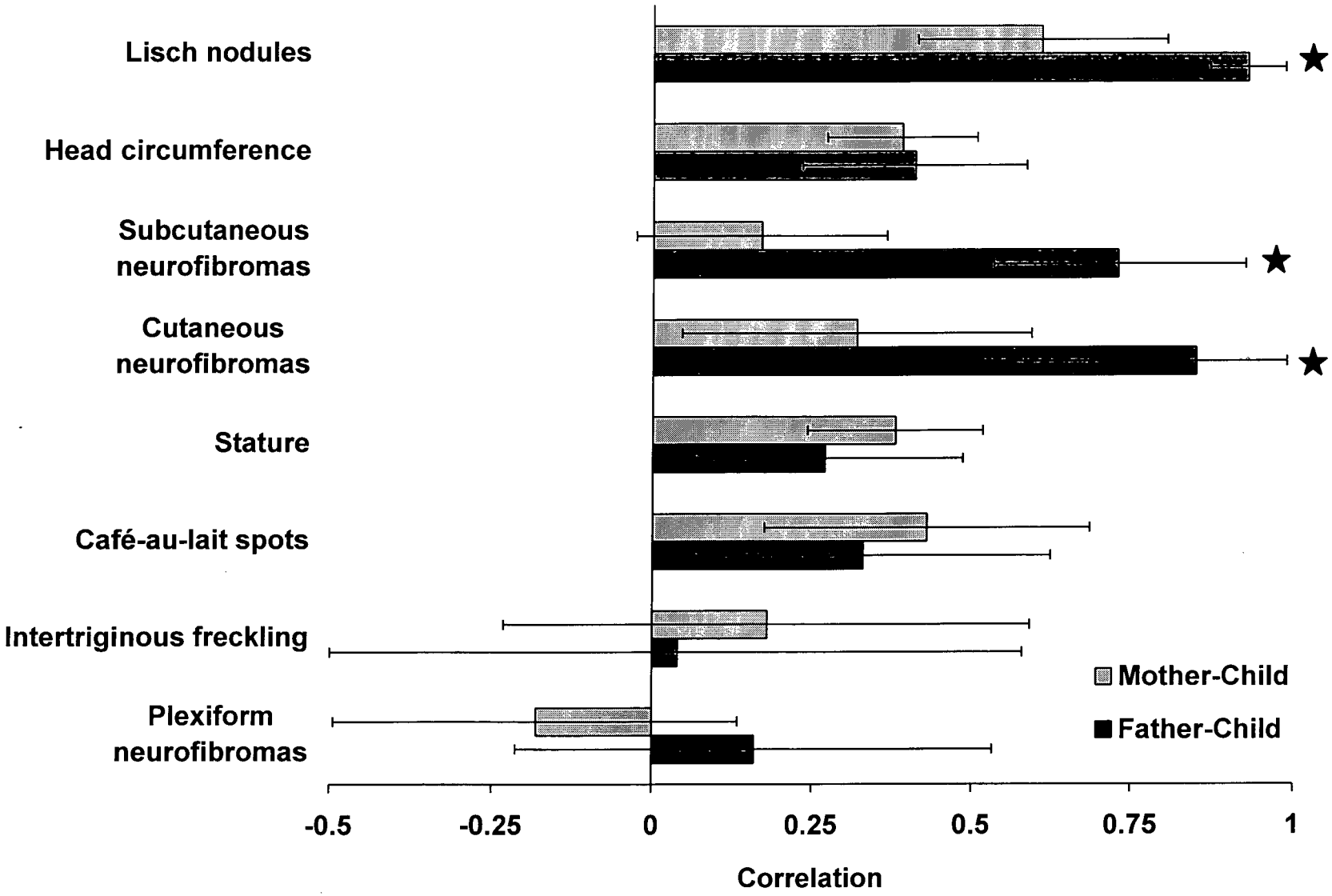

Lisch nodules
Head circumference
Subcutaneous neurofibromas
Cutaneous neurofibromas
Stature
Café-au-lait spots
Intertriginous freckling
Plexiform neurofibromas
Sib-Sib
Parent-Child
-0.5
-0.25
0
0.25
0.5
0.75
1
Correlation

Figure 7.4: Feature associations among mother- and father-child pairs with NF1. Adjusted intrafamilial correlation or latent correlation coefficients and 95% confidence intervals are shown for 8 features between 233 affected mother-child pairs and between 140 affected father-child pairs. A star indicates a significant difference between the coefficient between mother-child pairs and the coefficient between father-child pairs.


Lisch nodules
Head circumference
Subcutaneous neurofibromas
Cutaneous neurofibromas
Stature
Café-au-lait spots
Intertriginous freckling
Plexiform neurofibromas
Mother-Child
Father-Child
-0.5
-0.25
0
0.25
0.5
0.75
1
Correlation

children were found for 6 of the 8 features. Significant negative correlations were not observed for any of the features in either relationship.

Latent correlations between fathers and children are significantly greater than latent correlations between mothers and children for Lisch nodules (p=0.001), subcutaneous neurofibromas (p=0.0001) and cutaneous neurofibromas (p=0.02). Correlations or latent correlations do not differ significantly between father-child pairs and mother-child pairs for head circumference (p=0.85), stature (p=0.40), café-au-lait spots (p=0.62), intertriginous freckling (p=0.71) and plexiform neurofibromas (p=0.17).

7.6 Discussion

7.6.1 Variable expressivity in other disorders

Variable expressivity is a characteristic of many dominantly-inherited human genetic diseases and may have genetic or non-genetic causes. Possible genetic causes of variable expressivity include the effects of differences in the mutant allele, effects of the normal allele, and the effects of modifying genes. For example, analysis of phenotypic variation in von-Hippel-Lindau (VHL) disease has implicated unlinked modifying genes in the pathogenesis of ocular tumours (Webster et al. 1998). In another example the risk of ovarian cancer in *BRCA1* mutation carriers is modified by allelic variation at the unlinked *H-RAS* locus (Phelan et al. 1996). My study was designed to evaluate the relative importance of various genetic mechanisms in the interfamilial and intrafamilial variability of NF1.

7.6.2 Statistical significance

I analysed familial latent correlations for 10 NF1 clinical features treated as discrete variables and correlations for 2 NF1 clinical features treated as continuous variables, while adjusting for other related features, age and gender through statistical modelling. I found 7 of the features to have significant overall intra-familial associations (correlations or latent correlations) (Figure 7.1). I was also able to test for differences between associations among various classes of relatives for 8 of the 12 features studied. Differences between various classes of relatives were found for 6 of the 7 features with significant overall intra-familial associations (Figures 7.2-7.4).

Several features had significantly positive associations among 2nd degree relatives, but none were significantly greater than the associations for the same feature among 1st degree relatives (Figure 7.2). Similarly, several features had significantly positive associations between parents and children, but none were greater than associations for the same feature between sibs (Figure 7.3). I observed no significant negative associations, or positive associations that were greater in 2nd degree than 1st degree relatives or greater in parent-child pairs than among sibs. I would expect to observe such associations if chance were a major factor. This supports the statistical validity of my approach.

7.6.3 Representativeness of the sample

The NFDB draws its information from specialised clinics, so I was concerned about the representativeness of my sample. Furthermore, patients with unknown status of a feature were excluded from models involving that feature. Nevertheless, frequencies of

most features found among the familial cases used in this study (Table 7.1) are comparable to those found in another family study of variable NF1 expressivity (Easton et al. 1993). Cutaneous neurofibromas are twice as prevalent in the NFDB sample than in the sample used by Easton et al., due to a larger number of older patients in the latter study. Feature prevalences are also comparable to prevalences from two available population-based studies of NF1 patients (Samuelsson and Axelsson 1981; Huson et al. 1989a) (Table 1.3).

7.6.4 Comparison to other studies

Easton et al. (1993) studied 175 individuals with NF1 from 48 families, including 6 pairs of monozygotic twins, 76 pairs of sibs, 60 parent-offspring pairs, 54 2nd degree relative pairs and 43 3rd degree relative pairs. They examined 8 NF1 clinical features and found significant intrafamilial correlations for 3 quantitative variables: number of café-au-lait spots, number of cutaneous neurofibromas and head circumference. They also analysed 5 traits as binary variables, but these comparisons did not include adjustments for age. Furthermore, none of their analyses adjusted for the non-independence of multiple relative-pairs from the same family or of various clinical features.

My sample size is 5 times larger, and I examined 12 clinical features, 6 of which are the same as Easton's. Also, I included associations between features as covariates in the familial analyses. Unlike Easton et al., I did not have counts of café-au-lait spots and dermal discrete neurofibromas, but Easton's quantitative investigations of these features complement my binary analyses nicely. Both studies found are consistent with modifying gene influence on café-au-lait spots, but not on dermal discrete neurofibromas.

In all, 10 of my 12 features were treated as binary variables – I had quantitative data only on stature and head circumference. Many of the clinical features of NF1 (and other diseases) are by nature binary, and ours is the first study to examine associations for binary traits among different familial relationships while accounting for continuous covariates such as age. Similar methods have been used to study lens opacities (Framingham 1994) and liver cancer (Liang and Beaty 1991) in individuals who do not have NF1, but I may be the first to study an autosomal dominant disease in this manner.

7.6.5 Sample size considerations

Although this is by far the largest group of NF1 families ever studied, I only had 74 pairs of 2nd degree relatives. Models for most features used even fewer 2nd degree relatives because the data were incomplete. Subjects were included in a model only if the status of the modelled feature and all covariates was known (Table 7.3). These relatively small sample sizes are reflected in the wide 95% confidence intervals for the correlation and latent correlation coefficients among 2nd degree relatives (Figure 7.2). Furthermore, statistical techniques are less reliable for smaller sample sizes, so I must attach an additional note of caution to the point estimates for the correlation and latent correlation coefficients between 2nd degree relatives, particularly for Lisch nodules, head circumference, stature and intertriginous freckling, in which the analysis included 35 or fewer pairs of 2nd degree relatives (Table 7.3).

7.6.6 Minimising the confounding effect of age

The most important confounding factor in familial analyses of NF1 is age. Many disease features are more prevalent in older NF1 patients (Cnossen et al. 1998), and, if not appropriately controlled, age might produce an association between affected relatives of similar age (e.g., sibs) or obscure an association between relatives of very different ages (e.g., parents and children). My multivariate models minimise the confounding effect of age, but they may not eliminate this effect completely. The covariate representing age was significant in models for most features, but it is possible that a residual age effect is contributing to the observed differences between sib and parent-child pairs for features such as subcutaneous neurofibromas and intertriginous freckling that become more prevalent with age (Figure 7.3). Age is less likely to influence the intrafamilial associations for café-au-lait spots or plexiform neurofibromas, which, when considered as discrete variables, occur with a relatively stable frequency with age (Figures 4.1 and 4.5) (Riccardi 1992; DeBella et al. 2000b).

7.6.7 Statistical significance

I used one-tailed tests for 1st degree vs. 2nd degree and sib-sib vs. parent-child comparisons. Several of the results just reach a level of nominal statistical significance using one-tailed z-tests, and several others fall only a little short of doing so. Clearly these results require independent confirmation in future studies.

7.6.8 Interpretation of intrafamilial associations

Lisch nodules had significantly higher latent correlations among 1st degree relatives than among 2nd degree relatives (Figure 7.2). Higher latent correlations for 1st than 2nd degree relatives are consistent with effects produced by modifying genes at unlinked loci but might also result from environmental factors that are more likely to be shared among closer relatives. However, it is hard to imagine how specific environmental factors (such as viruses) could contribute to the development of Lisch nodules. Furthermore, sib pair and parent-child associations were similar for Lisch nodules (Figure 7.3), suggesting that environmental factors (at least those shared by sibs but not by parents and children) are not responsible for this association. No family studies have previously been done on Lisch nodules, and factors contributing to their development are unknown. My observations are consistent with the effect of a modifying gene on the pathogenesis of Lisch nodules.

Café-au-lait spots also had significantly higher latent correlations among 1st degree relatives than among 2nd degree relatives (Figure 7.2) – consistent with the influence of modifying genes. In addition, café-au-lait spots were more strongly associated between sibs than parents and children (Figure 7.3). Both sib pairs and parent-child pairs are 1st degree relatives who would be expected to share a similar proportion of non-allelic modifying genes. The differences I observed in latent correlations between sib pairs and parent-child pairs are unlikely to result from effects of modifying genes, unless dominant allelic variants are common at these modifying loci. If dominant allelic variants are common at these loci, then the sib-sib association is expected to be stronger than the parent-child association. Affected sibs would be expected to share the same

normal *NF1* allele by descent half of the time, but parent-child pairs rarely would. Effects of functional polymorphisms of the normal *NF1* allele might explain a higher latent correlation of these features among sib pairs than among parent-child pairs, but no direct evidence is available on this possibility, and the frequency of functional polymorphisms of the *NF1* locus is unknown. Interestingly, sharing of the normal *NF1* allele among sibs may also contribute to the difference between 1st and 2nd degree relatives observed for café-au-lait spots (Figure 7.2), since 1st degree relatives include sibs. The difference in association between sibs and parent-child pairs (Figure 7.3) is also consistent with the involvement of environmental factors shared by sibs. However, café-au-lait spots have a very early onset suggesting a limited role for environmental factors. Furthermore, no environmental trigger has been proposed for these lesions. Although I minimised the effect of age it could still contribute to stronger associations among sibs, who tend to be of similar age, than between parents and children, who differ greatly in age. However, the simulations shown in the appendix suggest that age was adequately controlled. Easton et al. (1993) found a higher correlation for café-au-lait spots between MZ twins than between sibs, suggesting the effect of a genetic locus or loci in addition to *NF1*. My findings of a strong latent correlation for café-au-lait spots in 1st degree relatives but no latent correlation among 2nd degree relatives are consistent with this interpretation.

Lisch nodules are melanocytic hamartomas that arise in iris tissue (Perry and Font 1982). Café-au-lait spots are pigmented macules composed of melanocytes with abnormally large pigment particles (Fitzpatrick 1981). Lisch nodules and café-au-lait spots share an origin from neural crest-derived tissue, but this is also true of some other

lesions characteristic of NF1, including neurofibromas of all types and intertriginous freckling (Bolande 1981). I previously reported an association between the occurrence of Lisch nodules and café-au-lait spots in individual NF1 patients (see Chapter 2), but intertriginous freckling was also associated – a feature that shows no indication of a stronger familial latent correlation among 1st degree than 2nd degree relatives (Figure 7.2). If the development of Lisch nodules and café-au-lait spots is influenced by modifying genes, it is unclear what the nature of these modifying factors is or whether they are the same or different for these two features. Associations between these features (Chapter 4) suggest that they share steps in pathogenesis. This hypothesis could be examined further by determining if the two features are transmitted together more often than expect by chance alone.

Intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas had similar latent correlations in 1st and 2nd degree relatives (Figure 7.2) but higher latent correlations between sibs than between parents and children (Figure 7.3). As stated above, both sib pairs and parent-child pairs share a similar proportion of non-allelic modifying genes, so the differences I observed in these latent correlations are unlikely to result from effects of modifying genes in the absence of dominance. Easton et al. (1993) found that concordance for dermal discrete neurofibromas (which include subcutaneous neurofibromas) between monozygotic twins was much higher than between sibs, an observation that suggests the involvement of a genetic factor. As stated above, affected sibs would be expected to share the same normal *NF1* allele half of the time, but parent-child pairs rarely would. Effects of functional polymorphisms of the normal *NF1* allele might explain a higher latent correlation of these features among sib pairs than among

parent-child pairs, but no direct evidence is available on this possibility, and the frequency of functional polymorphisms of the *NF1* locus is unknown. Another possible explanation is differences in environmental factors that are more likely to be shared among sibs than between a parent and child. Intertriginous freckling occurs in skin folds, and local environmental factors may play a role in the development of such freckling (Riccardi 1992). There is anecdotal evidence that subcutaneous neurofibromas may also develop as a result of trauma (Riccardi 1990). This hypothesis has not been tested formally, and it seems unlikely to account for the development of congenital diffuse plexiform neurofibromas. In any case, it is unclear why factors like cutaneous trauma would be more similar in sibs than in parent-child pairs.

Intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas and café-au-lait spots all share an origin from neural crest-derived cells. I found that café-au-lait spots and intertriginous freckling tended to occur together in individual NF1 patients, and so did cutaneous, subcutaneous, and plexiform neurofibromas, but associations were not seen between the features in these two groups (see Chapter 4). In the present study, I did not find a stronger latent correlation for cutaneous neurofibromas in sibs than in parent-child pairs, as I did for subcutaneous and plexiform neurofibromas (Figure 7.3).

Lisch nodules, subcutaneous neurofibromas, and cutaneous neurofibromas had higher latent correlations between affected fathers and children than between affected mothers and children (Figure 7.4). My sample included twice as many mother-child pairs as father-child pairs, so I was concerned about ascertainment bias. Perhaps mothers are more likely to bring children to the attention of an NF1 clinic that contributed data to the

NNFF International Database, whereas only severely affected father-child pairs tend to be seen in the NF clinics. However, the frequencies of all features studied were similar in affected fathers as in affected mothers and in their affected children (Table 7.1). Shared environment is unlikely to be the sole cause of associations between parents and children, due to large differences in age. It is also unlikely that shared environment is responsible for the difference in latent correlations between mother-child and father-child pairs. Likewise, a multifactorial influence with a more extreme threshold for males than for females cannot explain the observations for these features. Gender is not a significant predictive factor in any of my models (Table 7.2), and feature frequencies among affected daughters of affected fathers are similar to those among affected sons of affected mothers. Parent-of-origin effects on severity of NF1 have been suggested (Miller and Hall 1978; Hall 1981), but most studies do not support this possibility (Riccardi and Wald 1987; Huson et al. 1989a). One study found a male predominance among NF1 patients with pseudarthrosis but no significant parent-of-origin effect (Stevenson et al. 1999). Male-to-male inheritance is unlikely since gender is not a significant factor in any of my models (Table 7.2). Father-son concordances for Lisch nodules, subcutaneous neurofibromas, cutaneous neurofibromas are similar to father-daughter concordances, which argues against a Y-linked factor. My findings are consistent with a parent-of-origin effect on the strength of the parent-child association rather than with a more severe phenotype in affected offspring of parents of one gender when compared to affected offspring of parents of the other gender. Similar parent-child aggregation patterns have been reported for body mass index (Friedlander et al. 1988) and blood pressure (Hurwich et al. 1982) in the general population, but they are unprecedented in NF1. Such a pattern

could be caused by a triplet repeat that can expand when transmitted through mothers but not fathers. I do not know of any simple genetic mechanism that can explain this phenomenon in NF1 or for body mass index or blood pressure in the general population.

Head circumference and stature had similar correlations for all relationships. This suggests that the mutant *NF1* allele itself is most important in determining these correlations. Easton et al. (1993) also found evidence of the importance of the mutant allele in head circumference. The distributions of head circumference and stature in NF1 patients are unimodal (see Chapter 3), but both NF1 distributions are shifted relative to unaffected norms, suggesting that head circumference and stature are affected to a degree in all NF1 patients. Taken together with the results of the present study, it appears that the magnitude of this effect depends, at least partly, on the mutant *NF1* allele. The mutant *NF1* genotype also has a very strong effect on the phenotypic manifestations in patients with Watson syndrome (Allanson et al. 1991) or deletions of the whole *NF1* locus (Tonsgard et al. 1997; Dorschner et al. 2000).

7.7 Conclusion

The patterns of familial associations shown here suggest that genetic factors may be involved in determining the occurrence of various clinical features of NF1 and these factors may vary depending on the feature. In some instances, the mutant *NF1* allele may be most important. In other instances, the effects of the normal *NF1* allele or of unlinked modifying genes may predominate. More than one genetic factor may be involved, and the relative importance of various genetic and non-genetic effects may vary for different features.

8. GENERAL DISCUSSION

8.1 Summary

NF1 is a complex human disease with highly variable expressivity. *NF1* is a large gene whose functions are only beginning to be understood. It interacts in complex biochemical pathways involving Ras and protein kinase A (PKA), but the phenotypic consequences of *NF1* mutations exceed what can be attributed to *Ras* or *PKA*. The study of other complex human diseases has benefited tremendously from model systems or from in vitro biochemical experiments. However, humans are the only animal known to have a disease caused by heterozygosity for mutation at the *NF1* locus. Suitable animal models have been developed for a small number of NF1 disease features using chimeric double knockouts or double heterozygotes with specific mutations at other loci. These models are adequate for studying the occurrence of a few disease features, but there is currently no animal model that corresponds to most disease features found in humans.

In order to improve our understanding of the causes of variable expressivity in NF1, I used statistical methods to analyse the largest collections of NF1 clinical information in the world. In an effort to simplify the NF1 phenotype, I treated each of the features as if it were a trait segregating in NF1 patients. I have described how these "individual traits" relate to each other and, taking these relationships into account, how they aggregate among relatives with NF1.

8.1.1 Pair-wise analyses in individual NF1 patients

Replicable associations among common NF1 disease features in three independent databases (Chapters 2) strongly suggest that these features do not occur

entirely at random in NF1 and that some patients are more likely than others to develop particular features. This interpretation contrasts with the generally held view that any NF1 patient may develop any manifestation of the disease (Bernhart and Halperin 1990; Riccardi 1992). Most of the associations I observed have never been reported before, and their identification encouraged me to look for familial patterns of phenotypic aggregation.

A few of the individual clinical features were investigated further. Short stature and macrocephaly are well-recognised clinical features of NF1. The results in Chapter 3 suggest that these changes in growth affect all NF1 patients and are not limited to particular subgroups. These results suggest that stature and head circumference should be treated as continuous variables in future analyses. The mechanisms by which mutations of the *NF1* gene produce these phenotypic effects are unknown. Understanding such mechanisms may provide an important clue to the pathogenesis of more serious manifestations of NF1. *NF1* mutations in *Drosophila* result in a smaller phenotype through the protein kinase A pathway (Guo et al. 1997; The et al. 1997) and human neurofibromin also has protein kinase A binding sites (Marchuk et al. 1991; Fahsold et al. 2000), but their role, if any, in growth is unknown.

My findings in Chapter 6 suggest that the occurrence of cutaneous neurofibromas in NF1 patients is not influenced by the local presence of diffuse plexiform neurofibromas. In fact, all three of the lesions studied in this chapter (café-au-lait spots, cutaneous neurofibromas, and plexiform neurofibromas) occurred independently of each another in almost all of the body segments analysed (Table 6.2).

8.1.2 Analyses of several different features at once

The analyses in Chapters 2 and 6 considered only two features at a time in individuals. I examined the relationship among several different features at the same time and summarised the results in Chapters 4 and 5. In these analyses, I observed several associations among clinical features. It is not easy to pool the results of the 13 separate models I generated into a single conceptual framework of interdependencies of clinical features in NF1. Nevertheless, the associations I observed are consistent with the existence of three groups of features among the 13 features studied (Figure 4.14) – pigmentary changes, neurofibromas and CNS findings. All features belonging to a group must be taken into account to best describe the occurrence of other features belonging to the same group. Subjects who have one or more of a group's features may be fundamentally different from those who do not have any of a group's features.

Each group may share particular pathogenic elements that differ between groups. For instance, patients who tend to have pigmentary changes may have melanocytes with a particular neurofibromin activity profile – a different profile than those who tend not to have pigmentary changes. The melanocytes in the affected group might be more susceptible to limited proliferation in response to heat or friction. Alternatively, the effect could be on splicing of a particular isoform of neurofibromin or on the level of neurofibromin expression. Analogous differences may exist in the Schwann cells of patients who tend to have neurofibromas or in the glial cells of those with CNS features. Each of these effects could be due to several different factors at the *NF1* locus or at unlinked loci. Subsequent analyses attempted to differentiate between various genetic causes and are discussed below.

The mechanisms shared by associated features may be different and independent for each group of features. This is supported by the observation that no feature, except Lisch nodules, was found to belong to more than one group. In terms of pathogenesis, this means that a single factor or set of factors could account for each of the three groups of features, but one factor is unlikely to account for all three groups of features. These NF1 features are not mutually exclusive, and many patients belong to more than one group.

Logistic regressive models in individuals were also used to investigate an NF1 clinical feature of special interest – unidentified bright objects on MRI (UBOs). My observations in Chapter 5 suggest that UBOs and other NF1 clinical features do not occur independently and may be pathogenetically related. The common thread between the associated features (optic gliomas, other neoplasms, Lisch nodules, and subcutaneous neurofibromas) may be dysregulated cellular proliferation resulting from haploinsufficiency of neurofibromin (Gutmann et al. 1999; Ingram et al. 2000).

Analyses of individual patients (Chapters 2, 4-6) suggest that shared pathogenetic mechanisms underlie several common features of NF1. If genetic factors influence these pathogenetic mechanisms, one would expect familial aggregation of such features to occur.

8.1.3 Familial analyses

Preliminary familial analyses in Chapter 2 suggest that some clinical features tend to aggregate in affected parents and children. The age-adjusted number of affected body segments for all three of the lesions studied in Chapter 6 (café-au-lait spots, cutaneous

neurofibromas, and plexiform neurofibromas) was correlated among relatives with NF1. These significant intrafamilial associations are consistent with other evidence that familial factors contribute to the development of cutaneous neurofibromas and café-au-lait spots in patients with NF1 (Easton et al. 1993).

Taking local and familial analyses into consideration, multiple factors appear to be involved in the pathogenesis of both plexiform and cutaneous neurofibromas as well as of café-au-lait spots. Some of these factors may to be genetic, but others are not. The intrafamilial correlations I observed were statistically significant, but they were also moderate in magnitude. Even the twins reported by Easton et al. (1993) were not perfectly concordant for plexiform neurofibromas and café-au-lait spots. Non-familial and non-genetic factors must also influence the occurrence of these lesions.

The analyses reported in Chapters 2 and 4 suggest that individual features should not be analysed independently, but that other features, as well as age, must be taken into account. With this in mind, familial analyses were extended to several different types of familial relationship, and the results were used to evaluate the different familial mechanisms that could be contributing to NF1 variable expressivity.

The patterns of familial associations summarised in Chapter 7 suggest that genetic factors involved in determining the occurrence of different clinical features of NF1 vary. This conclusion is based on the genetic similarities and differences between different classes of affected relatives. Head circumference and stature had similar correlations among all classes of affected relatives and may be influenced by the type of mutation at the *NF1* locus. Lisch nodules and café-au-lait spots had higher latent correlations among 1st degree relatives than among 2nd degree relatives and so may be influenced by other

unlinked loci. Intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas and café-au-lait spots had higher latent correlations among sibs than parents and children and so may be influenced by: variations in the normal *NF1* allele, dominant alleles at modifying loci or shared environment. These interpretations are corroborated by the results from an equivalent analysis of simulated data (Appendix). More than one genetic factor may be involved, and the relative importance of various genetic and non-genetic effects may vary for different features.

8.2 How this study fits into current NF1 research

NF1 research today is proceeding in two opposite but complementary directions. On the one hand, researchers are trying to investigate the natural history and pathogenesis of individual features. The aim of such studies is to develop treatment strategies for individual features and their complications. On the other hand, molecular biologists are trying to understand how the *NF1* gene and its product, neurofibromin, work. Presumably neurofibromin interacts with Ras via its GRD and with PKA via its CSRD, a newly discovered functional domain. However, both of these pathways are complex and dynamic and the cellular consequences of *NF1* mutations are largely a mystery. Clinical and molecular approaches ultimately hope to address the same question – how do defects in *NF1* gene result in the myriad phenotypes of neurofibromatosis 1?

Indeed the NF1 phenotype may be too complex to examine all at once. Previous attempts to assign a single severity score to a patient have not borne much fruit (Carey et al. 1979; Riccardi 1992). Such scales are inevitably subjective and may depend too much on stochastic factors such as where a tumour is located on the body. My study suggests

that some clinical features tend to be found together in the same patients. In addition, and perhaps most importantly, age must be taken into account. Due to their progressive nature, many NF1 features are more prevalent in older age groups. A severity scale relative to other patients of similar age would be much more meaningful than one that ignores age.

This study describes how various clinical features of NF1 are related and what genetic mechanisms could be involved in their development. This may provide clues to researchers on both clinical and molecular fronts. Clinical researchers can get a more quantitative idea of which patients are at higher risks for the development of specific disease features. Molecular biologists can get a better idea of what genetic mechanisms are likely to play a part in the pathogenesis of particular features. For instance, I found that plexiform neurofibromas are more common in patients with subcutaneous neurofibromas. Although these results should not be used to make predictions in individual patients, they may stimulate interest in the natural history of neurofibromas. They may also spur researchers to compare patients who have both plexiform and subcutaneous neurofibromas and contrast them to those who lack these features.

8.3 Future directions

The results of this study provide clues to the pathogenesis of several common clinical features of NF1. In addition, the methods used in this study can be applied to other complex disorders, particularly those with notable intra-familial variability. One of the initial goals of this study was to generate age-specific risks for the development of several NF1 clinical features, taking into account covariates such as gender and the

presence or absence of other associated clinical features. The cross-sectional nature of the available data precludes reliable risk estimates. Risk estimates can be gleaned only from longitudinal studies, but a long and costly follow-up study of a large cohort of NF1 patients has been difficult to justify until now. My observations of several associations among NF1 features in individuals and among relatives go a long way in helping to justify long-term longitudinal studies of NF1.

NF1 allelic heterogeneity is one hypothesis to explain the results presented here. Many NF1 patients have been genotyped at the *NF1* locus. However, phenotypic information has not been collected in a consistent manner. A standard phenotyping protocol would allow the data to be pooled and enable testing for genotype-phenotype correlations. It would be important to emphasise primary clinical features rather than their consequences. Riccardi has long espoused the need for this distinction (Riccardi 1992). For example, the pathogenesis of scoliosis depends on whether or not it is caused by spinal neurofibromas. Such distinctions are not made in the current databases, and the heterogeneity of this feature may help to explain why I found no associations involving scoliosis in individuals or families. Clearly, features such as neoplasms, pseudarthrosis and scoliosis are of great concern to patients and their physicians, but other, often less threatening features, like Lisch nodules or café-au-lait spots, may also provide valuable insights into *NF1* function and disease pathogenesis. They should not be neglected during phenotyping.

The results presented here suggest that the majority of the features studied (and most of the cardinal clinical features) are familial and may have a genetic component. The patterns of variable expressivity are subtle, so data would be required on a large

number of patients. An objective measurement such as a count of these lesions (e.g. number of neurofibromas) would enable a more detailed analysis of familial segregation patterns and would require a smaller number of patients than binary variables of the type used in most of the studies reported here. Given the progressive nature of many NF1 disease features and the potentially confounding effects of age on analysis, it is also essential that the data be representative of all age groups.

These results show that much of the phenotypic variability in NF1 may be due to genetic factors unlinked to the NF1 locus. Neurofibromin interacts with Ras and possibly PKA. Segregation analysis is typically used to identify the number of other genes involved and their modes of inheritance. Segregation analysis for possible modifying genes was previously performed in a small sample of NF1 patients by Easton et al. (1993), but the results were inconclusive. Segregation analysis with the data used in the present study may be problematic for several reasons. The data are binary and segregation analysis methods are not as robust for binary variables as they are for continuous or polytomous variables (Khoury et al. 1993). Most NF1 clinical features are highly dependant on age and do not occur independently of one another. I have attempted to minimise these effects, but they may be harder to control in more complex models.

With the completion of the Human Genome Project, genetic markers throughout the genome are available – one study reports 1.42 million single nucleotide polymorphisms (SNPs), providing one SNP for every 1.9 kilobases. (Sachidanandam et al. 2001). Over 80% are polymorphic at frequencies above 10%. Although a genome scan for modifying loci is theoretically possible, it is currently impractical to genotype

NF1 patients at hundreds of thousands of markers distributed throughout the genome and test if the markers are associated with specific clinical features or groups of features.

Mouse and *Drosophila* models have been helpful in the understanding of neurofibromin biochemical interactions and pathogenesis of features such as malignant peripheral nerve sheath tumours (MPNSTs) and astrocytomas. Astrocytomas occur in 15% of NF1 patients, and MPNSTs affect up to 10% (see section 1.5). Far more common are problems related to dermal discrete neurofibromas or bony abnormalities. Animal models of these and other common features would enable studies into their pathogeneses.

NF1 is disorder well known for its highly variable expressivity. The results of my study refine this perception: it is a disorder which also has subtle but definite associations among its features both in individuals and among affected relatives. An ardent search for putative genetic factors involved in variable expressivity of the NF1 phenotype assumes that such factors exist, but the evidence supporting this assumption has hitherto been scant. My study suggests that genetic factors *are* involved in phenotypic variability in NF1. Multivariate probit analyses has been used for perhaps the first time in a study of this type. I have applied them to both real and simulated data. These methods can also be applied to other human disorders. NF1 may affect the human species exclusively, but it is not the only complex human disorder that lacks understanding. It may be very useful to perform genetic epidemiological studies of complex human disorders in addition to molecular investigation. I hope my findings encourage other investigations of NF1 and other complex human disorders. Ultimately, the answers will rely on molecular genetic experiments, and I am eager to see whether they will confirm my conclusions.

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APPENDIX – ANALYSIS OF SIMULATED DATA WITH MPROBIT

Introduction

The results of multivariate probit (MPROBIT) (Joe 1997; Joe 2000) analysis have been used in this study to support the involvement of genetic factors in the phenotypic variability of NF1. However, MPROBIT is newly revised for biostatistical analysis in this study; it has never before been used to analyse pedigree data. I wanted to assess the validity of this method. To that end, I have simulated a monogenic disease with five different features, each with different genetic component. I then used MPROBIT to analyse each feature and checked if the intra-familial latent correlation pattern found for each feature was consistent with the genetic components used to simulate them.

Subjects and Methods

The Genometric Analysis Simulation Program (GASP) is a software tool that can generate samples of family data based on user specified genetic models (Wilson and Sorant 2000). I used GASP to simulate a sample of pedigrees similar to the ones described in the National Neurofibromatosis Foundation International Database (NFDB). The simulated disease is caused by one 12-allele locus – 10 different dominant disease-causing alleles, each with a frequency of 0.01, and two different normal alleles, each with a frequency of 0.45. This is similar to NF1, where there are many rare mutant alleles and only a few common normal alleles are known. Five different features were generated simultaneously with the disease. (1) Random: a random feature was generated by finding the inverse of the standard normal cumulative distribution of random numbers between 0 and 1 inclusive. (2) Mutant: 60% of the variation in this feature is due to the mutant alleles at the disease locus, and 40% of the variation is due to random effects. (3)

Polygenic: 90% of the variation in this feature is due to a polygenic component, and 10% of the variation is due to random effects. The polygenic component for parents is generated using a random deviate from a normal distribution. The polygenic component for children is based on mid-parental value and a random deviate from a normal distribution. (4) Unlinked: 90% of the variation in this feature is due to a single locus unlinked to the disease locus, and 10% of the variation is due to random effects. The unlinked locus has two alleles with equal frequencies but opposite effects. One homozygous genotype increases susceptibility, the other homozygous genotype decreases susceptibility and the heterozygous genotype has no effect. (5) Wild: 90% of the variation in this feature is due to the normal alleles at the disease locus, with 10% of the variation due to random effects.

Prevalence of most NF1 features is typically greater than zero at birth, increases with age and is less than 100% late in life. Although these prevalence by age curves are typically non-linear, they can usually be represented as linear if age is transformed using a logarithmic function. Each of the features was simulated as a continuous liability that was then compared to an age-specific threshold (Figure A.1). The same threshold was used for all five features. For convenience, this threshold for each age was the z-score corresponding to a probability of $1- e^{-10/(age + 4)}$. The final derived feature was defined as "present" if its normally distributed genetic liability exceeded the age-specific threshold. No parent of origin effect was included in these models.

One sample of 4995 individuals in 555 families was generated. Each simulated family has two parents, three offspring, two aunts or uncles and two grandparents. NF1 is inherited as a dominant disease and mutant homozygotes are thought not to be viable.

Therefore only the 820 subjects in 244 families with exactly one mutant allele were used in the subsequent analysis. These include 277 affected sib pairs, 255 affected mother-child pairs, 271 affected father-child pairs and 244 pairs of affected 2nd degree relatives. MPROBIT was used to estimate intra-familial latent correlation coefficients for each of the five features, while minimising the confounding effects of age (see section 7.4.4).

Results

Figure A.2 shows the adjusted intrafamilial latent correlation coefficients and their 95% confidence intervals for each of the five features among all 820 affected relatives with NF1 from the 244 families studied. In these estimates, all relatives are treated the same regardless of relationship. Statistically significant positive intrafamilial latent correlations were observed for all of the features except the random feature.

Figure A.3 shows the adjusted intrafamilial latent correlation coefficients and 95% confidence intervals for the five clinical features among affected 1st and 2nd degree relatives. Statistically significant positive latent correlations between 1st degree relatives were found for all of the features except the random feature. A significant positive latent correlation between 2nd degree relatives was also found for the feature influenced by the mutant allele. Significant negative latent correlations were not observed for any of the features. Latent correlations were significantly greater among 1st degree relatives than among 2nd degree relatives for features influenced by the normal allele ($p=0.0008$), a polygenic component ($p=0.03$), and a single unlinked locus ($p=0.006$). Latent correlations among 1st degree relatives were not statistically different from latent

correlations among 2nd degree relatives for the random feature (p=0.47) or the feature influenced by the mutant allele (p=0.18).

Figure A.4 shows the adjusted intrafamilial latent correlation coefficients and 95% confidence intervals for the five features among affected sib pairs and affected parent-child pairs. Statistically significant positive latent correlations between sibs were found for all but the random feature. Significant positive latent correlations between parents and children were found for three features. Significant negative latent correlations were not observed for any of the features. Latent correlations were significantly greater between sibs than between parents and children for the feature influenced by the normal allele ($p<0.0001$) Latent correlations between sibs were not statistically different from the latent correlations between parents and children for features influenced by an unlinked locus (p=0.22), a polygenic component (p=0.38), the mutant allele (p=0.50) or the random feature (p=0.44).

Figure A.5 shows the adjusted intrafamilial latent correlation coefficients and 95% confidence intervals for the five features between affected mother-child pairs and between affected father-child pairs. Statistically significant positive latent correlations between mothers and children were found for three of the features. Significant positive latent correlations between fathers and children were also found for the three features. Significant negative latent correlations were not observed for any of the features in either relationship. Latent correlations do not differ significantly between father-child pairs and mother-child pairs for any of the features: the feature influenced by the normal allele (p=0.17), an unlinked locus (p=0.94), a polygenic component (p=0.29), the mutant allele (p=0.92) or the random feature (p=0.37).

Conclusion

Statistically significant intra-familial latent correlations were observed for the four features with genetic components, but not for the random feature. Higher latent correlations for 1st than 2nd degree relatives were observed for the features whose major genetic component was one or more modifying genes at unlinked loci, but not for any other features. A similar latent correlation for all relationships was observed for the feature with a normal allele component. This suggests that genetic components can result in significant intra-familial latent correlation coefficient estimates using MPROBIT. Furthermore, these latent correlation coefficient estimates are consistent with the simulated genetic components underlying the disease features.

Figure A.1: Prevalence by age of a simulated clinical feature.
This feature was used to generate an age-specific liability threshold. Prevalence of most NF1 features is typically greater than zero at birth, increases with age in a non-linear manner and is less than 100% late in life. Each of the five features was initially simulated as a continuous liability. Each of their continuous liabilities was compared to an age-specific threshold. The final derived feature was defined as "present" if its normally distributed genetic liability exceeded the age-specific threshold.

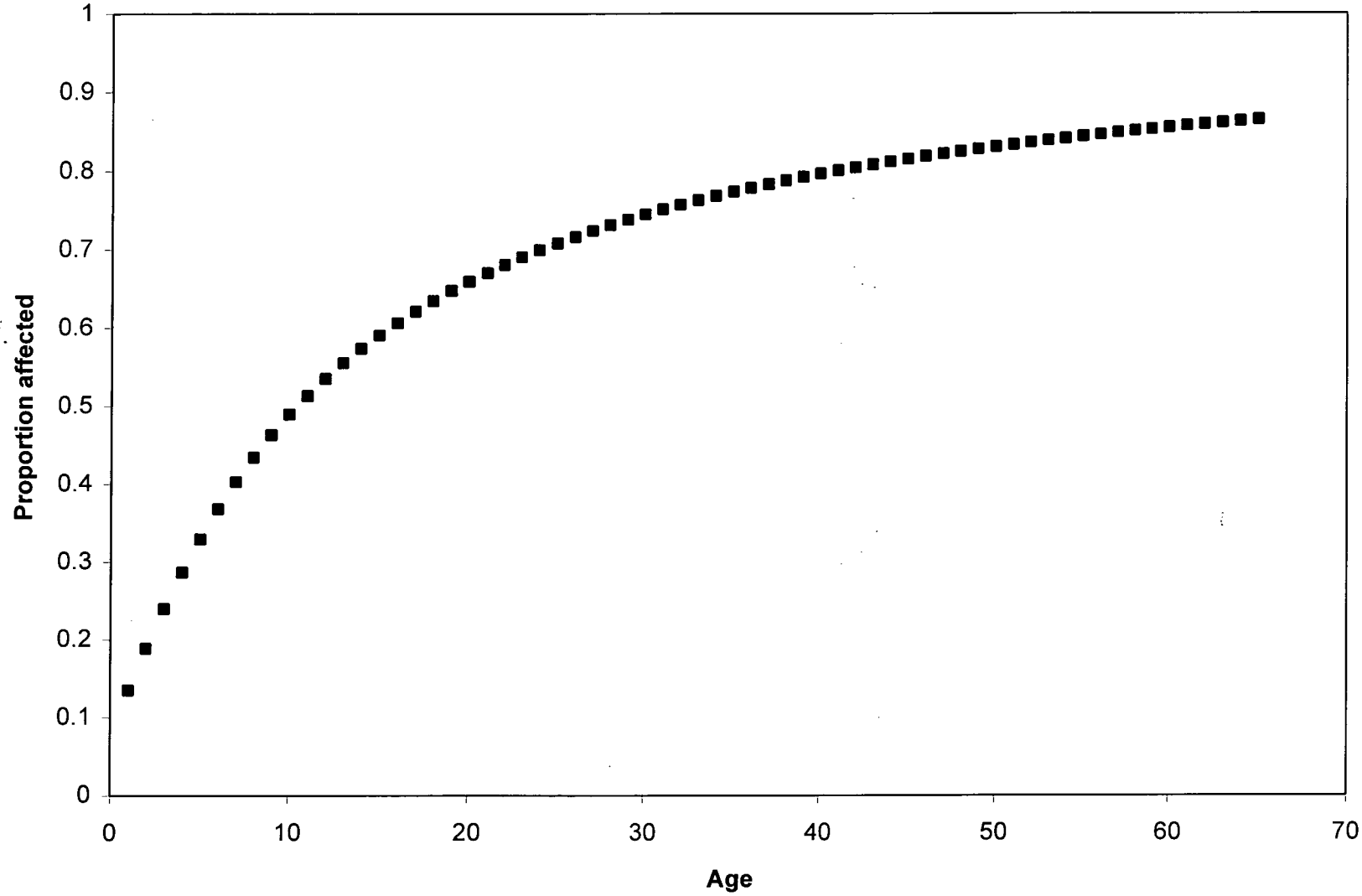

Proportion affected
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Age

Figure A.2: Associations of simulated features among all affected relatives. Adjusted intrafamilial latent correlation coefficients and their 95% confidence intervals are shown for each of the five features among all 820 relatives with NF1 from the 244 families simulated. In these estimates, all relatives are treated the same regardless of relationship.

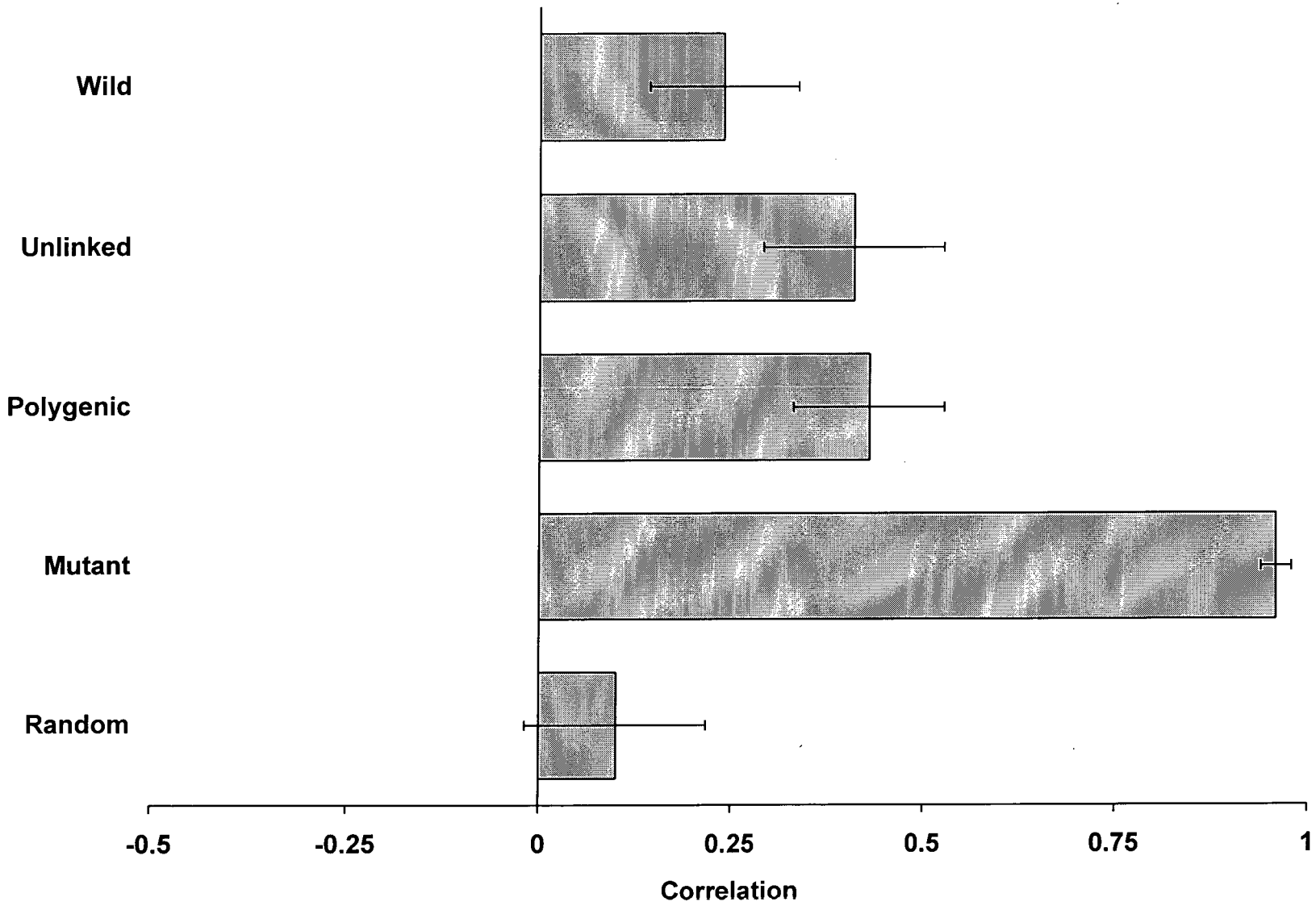

Wild
Unlinked
Polygenic
Mutant
Random
-0.5
-0.25
0
0.25
0.5
0.75
1
Correlation

Figure A.3: Associations of simulated features among 1st and 2nd degree relatives. Adjusted intrafamilial latent correlation coefficients and 95% confidence intervals are shown for five clinical features among affected 1st degree and 2nd degree relatives. A star indicates a significant difference between the coefficient among 1st degree relatives and the coefficient among 2nd degree relatives.

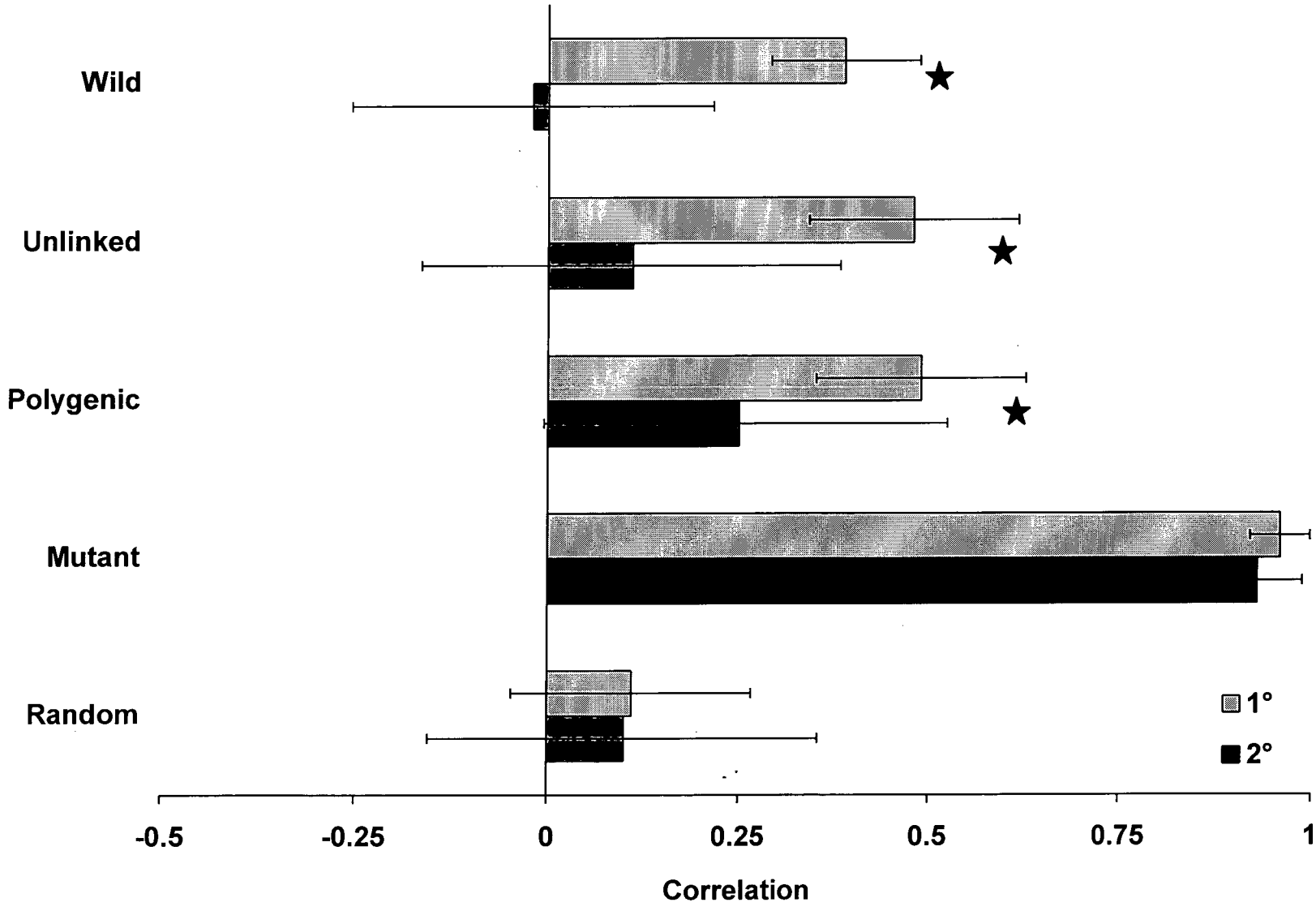

Wild
Unlinked
Polygenic
Mutant
Random
1°
2°
-0.5
-0.25
0
0.25
0.5
0.75
1
Correlation

Figure A.4: Associations of simulated features among sib and parent-child pairs. Adjusted intrafamilial latent correlation coefficients and 95% confidence intervals are shown for five features among affected sib pairs and among affected parent-child pairs. A star indicates a significant difference between the coefficient among sibs and the coefficient among parents and children.

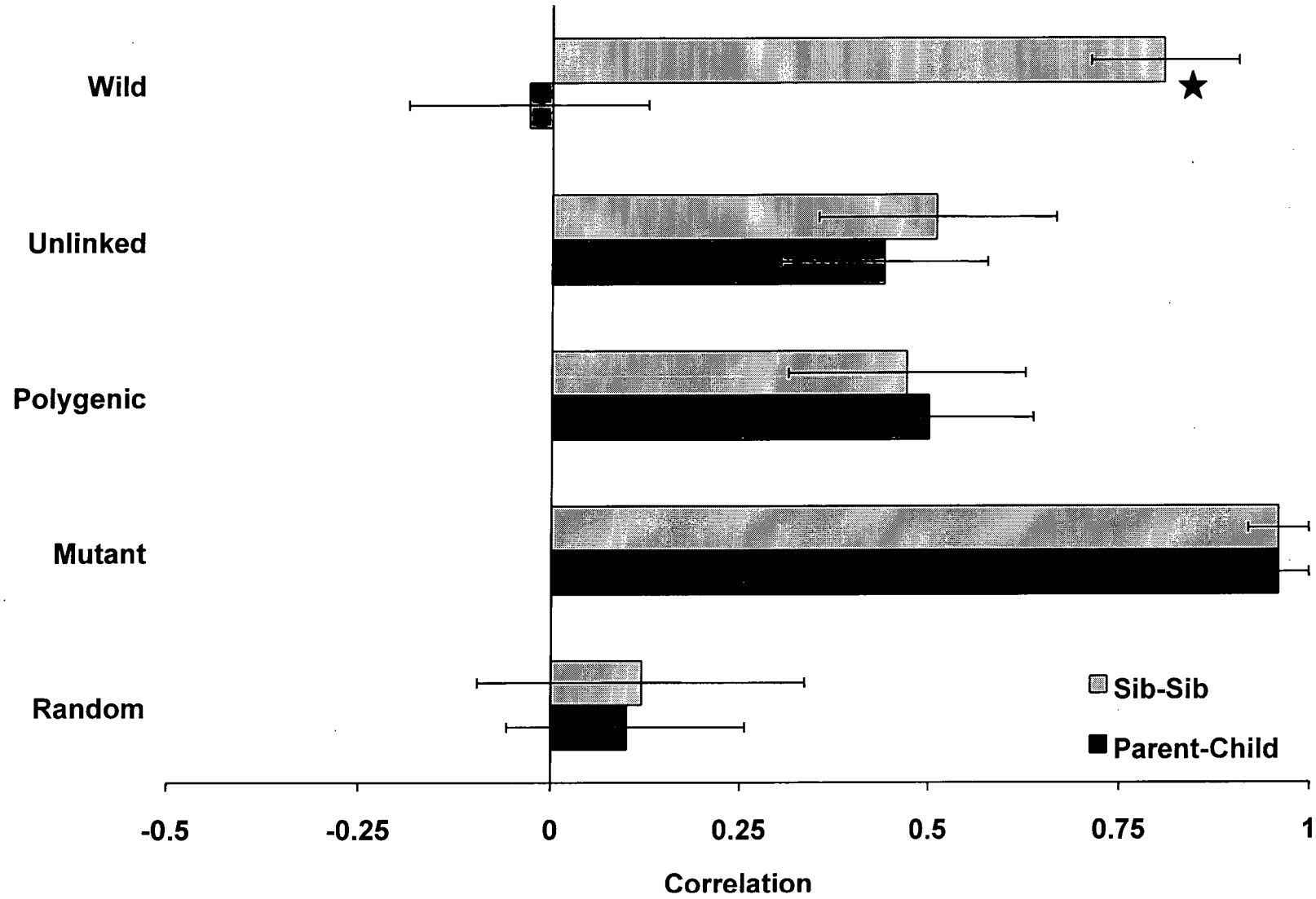
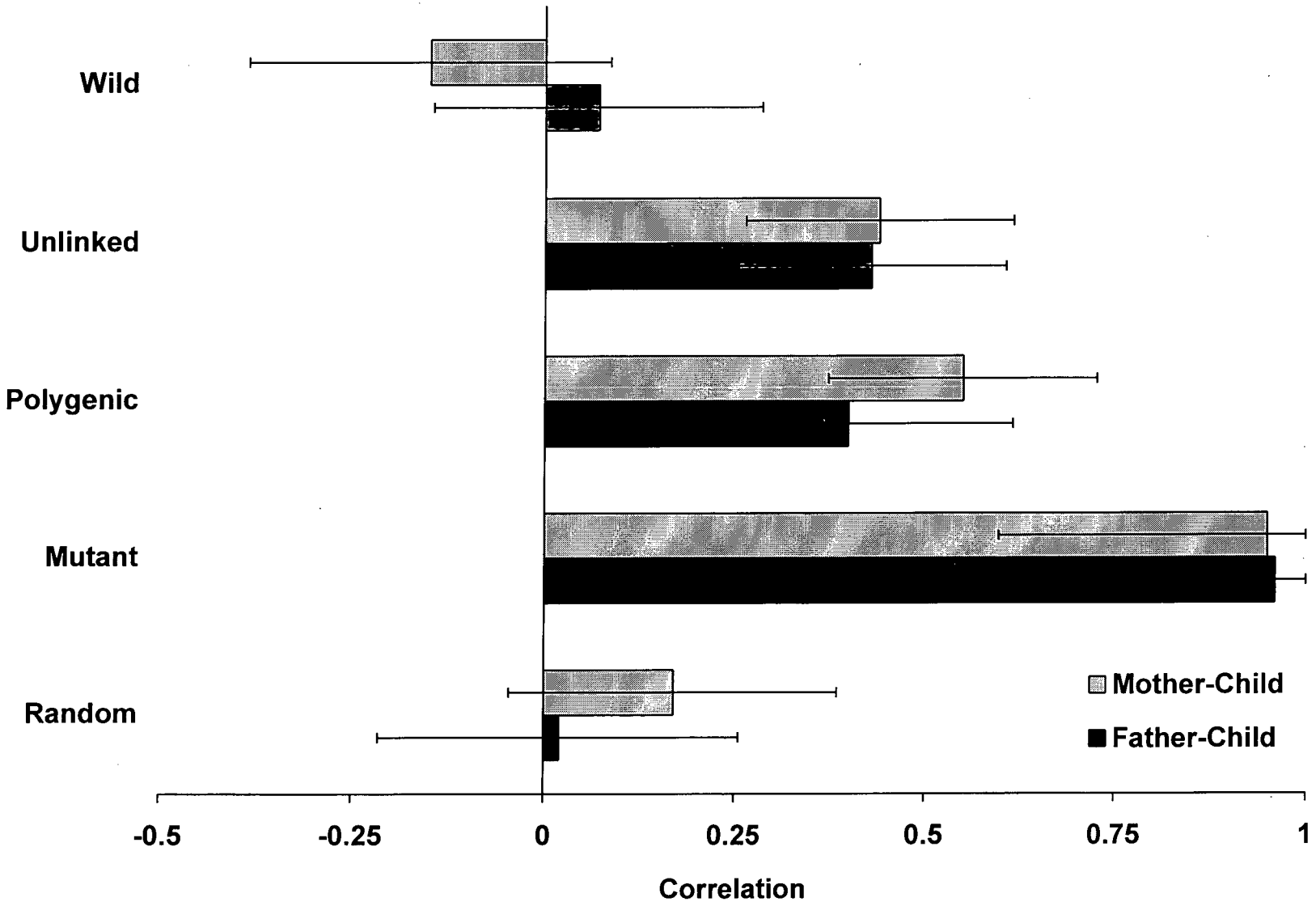

Wild
Unlinked
Polygenic
Mutant
Random
Sib-Sib
Parent-Child
-0.5
-0.25
0
0.25
0.5
0.75
1
Correlation

Figure A.5: Associations of simulated features among mother- and father-child pairs.
Adjusted intrafamilial latent correlation coefficients and 95% confidence intervals are shown for five features between affected mother-child pairs and between affected father-child pairs.


Wild
Unlinked
Polygenic
Mutant
Random
Mother-Child
Father-Child
-0.5
-0.25
0
0.25
0.5
0.75
1
Correlation