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13. ABSTRACT *(Maximum 200 Words)*

This project is designed to characterise the sources of phenotypic variability in neurofibromatosis 1 (NF1) by a combination of clinical, statistical, epidemiological, and molecular genetic methods. During the second year of the project, we analysed associations found in clinical and genetic data from the NNFF International Database with logistic regression, generalized estimating equations. During the third year, the results of these methods were extended to use multivariate probit models on familial data. These are novel statistical techniques that have not been used in this way before and they yielded interesting results about the sources of phenotypic variability in NF1. We were able to determine that the presence of some features of NF1 are more influenced by variability in the NF1 allele, others by the normal NF1 allele and still others by unlinked modifying genes.

We have asked for and have received a fourth year, no cost extension to complete the analysis of NF1 allele-phenotype associations and to continue to explore some other possible sources of variability, such as imprinting.

14. SUBJECT TERMS
Neurofibromatosis 1(NF1), assocations, pathogenesis, NF1 allele, genotype-phenotype correlation.

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Introduction

The natural history of neurofibromatosis 1 (NF1) is incompletely understood. Although NF1 is progressive over the course of an affected individual's life, the rate of progression and the occurrence of serious complications vary greatly. The manifestations of NF1 are extremely variable in different patients of the same age, different affected members of a single family, and even a single affected individual at different times in life. The purpose of this project is to characterise the sources of phenotypic variability in NF1 through a combination of clinical, statistical, epidemiological, and molecular genetic methods. This report covers the third year of the project.

Annual Report -body

Technical objectives 1 and 2: Identification of associations between features of NF1.

Task 1: Identify associations of features within probands by screening the 5000 possible pair-wise associations on both databases.

This task was completed during the first year of the project. (Please see year one Annual Report; Szudek et al., 1999a [Appendix] for details.)

Task 2: Identify associations of NF1 features between related individuals (e.g. parent-child diads) in both databases.

This task was completed during the first year of the project. (Please see year one Annual Report and Szudek et al., 1999A [Appendix] for details.)

Task 3: Examine apparent associations (from task 1) in detail for other covariates or confounding factors, using log-linear models for binary traits and stratification for age or other continuous variables.

This task was completed during year 2 of the project . (Please see year 2 Annual report and Szudek et al, submitted for publication, [Appendix] for details).

Task 4: Perform detailed examination of apparent familial associations identified in task 2.

This task was completed during year 2 of the project . (Please see year 2 Annual Report and Szudek et al, *Am J Hum Genet* 67(Suppl. 2):211, 2000 , [Appendix] for details)

Technical objective 5: Determine the contribution of genetic and non-genetic factors to the presence or absence of certain NF1 traits.

Task 5: For traits showing familial aggregation (from task 2), partition variance by using various techniques including logistic regressive modelling and multivariate normal methods.

This task was begun during year 2 of the project and completed this year. (Please see year 2 Annual Report, and Szudek et al *Am J Hum Genet* 67(Suppl. 2):211, 2000 for details).

We have continued the familial analysis with multivariate probit models. We compared

odds ratios between parent-child pairs, sib, and pairs of second-degree relatives in those features found to be familial (See Szudek et al *Am J Hum Genet* 67(Suppl. 2):211, 2000). Three distinct trends were observed among the correlations for familial features:

1) Some features, such as macrocephaly, had similar correlations for affected family members regardless of how closely they were related. This implies a major effect of the mutant *NF1* allele.
2) Other features, such as Lisch nodules, had significantly higher correlations between affected first-degree relatives (parent-child and sib-sib) than among second-degree relatives. This pattern is consistent with an effect of unlinked modifying genes.
3) Features such as sub-cutaneous neurofibromas had significantly higher correlations between affected sibs than between affected parents and children. This pattern suggests an influence of the normal *NF1* allele on the phenotype observed.

We are now using these multivariate methods to look for an effect of imprinting. This approach provides a robust way to test for differences in the observed severity of clinical features in mother-child pairs and father-child pairs with NF1.

Technical objective 6: Identify allele-phenotype correlations between "familial phenotype" and allele type.

Task 1a: Set up techniques for strategic screening process for identification of constitutional mutations of NF1.

This task was completed during the first year of the project. (Please see year one Annual Report; Szudek et al., 1999a [Appendix] for details.)

Task 7 (Vancouver): Identify 40 patients for mutation identification from familial phenotypes identified in task 2. Contact contributing centres and obtain blood samples.

and

Task 2a (Salt Lake City): Identify mutations in these 40 NF1 patients.

Six phenotypes that were found to be familial were chosen for molecular analysis. These six phenotypes were described in detail in the year 2 report. During year 3, we have been working with the physicians who originally contributed clinical data to the NNFF International Database to verify the clinical phenotype and obtain a blood sample on each patient selected for mutation analysis. This is a time-consuming and occasionally frustrating experience because all requests for clinical updates and blood samples must be made through the contributing physician rather than by direct patient contact. The participation of all of these physicians is voluntary – they receive no compensation for contributing data to the database or for bringing the patients back for further studies, both of which take time away from clinical practices. Nevertheless, we have

now obtained, or are in the process of obtaining, specimens from NF1 patients with five of the phenotypes of interest:

Large deletion phenotype	11 patients
Malignant peripheral nerve sheath tumours	6 patients
NF1 vasculopathy	5 patients
Optic glioma and other central nervous system gliomas	2 patients
Late-diagnosed NF1, an initially mild phenotype	7 patients

Clearly, several of the categories need substantially more patients to enable us perform appropriate inter-group comparisons of phenotype. For this reason, we requested and were granted a one year no-cost extension to this grant. We are concentrating our efforts in locating and obtaining specimens for the final phase of this grant. As described in the previous report, the molecular protocol has been defined and tested and we are confident that the samples can be run quickly once they have been received in Dr. Viskochil's lab.

Task 6: Review all cases with "familial phenotypes" in the NF1 Genetic Analysis Consortium Database and the published literature, looking for common mutations or mutation types.

Please see comment in year 2 Annual Report.

Key Research Accomplishments (years 1, 2, and 3)

- Demonstration of associations among clinical features in NF1 patients using stratified Mantel-Haenszel analysis and logistic regression to control for the confounding effect of age.
- Demonstration of associations of NF1 clinical features among family members using logistic regression and multivariate probit models to control for the confounding effect of age.
- Identification of familial phenotypes as candidates for allele-phenotype correlations.
- Development and validation of exhaustive molecular screening process for pathogenic mutations of the *NF1* locus.
- *Testing of NF1 patients with candidate familial phenotypes for allele-phenotype correlations is currently in progress.*

Reportable Outcomes

1. **Manuscripts, abstracts and presentations**:

Papers published in peer-reviewed scientific journals

Baser ME, Birch PH, Evans GR, Friedman JM. Association of superficial plexiform and paraspinal neurofibromas in neurofibromatosis 1. *Neurology* Apr 22;52(7):1519-20, 1999.

DeBella K, Szudek J, Friedman JM. Use of the NIH criteria for diagnosis of NF1 in children. *Pediatrics* 105:608-614, 2000.

DeBella K, Poskitt K, Szudek J, Friedman JM. Use of unidentified bright objects on MRI for diagnosis of neurofibromatosis 1 in children. *Neurology* 54: 1646-1651, 2000.

Friedman JM. Epidemiology of NF1. *Am J Med Genet* 89:1-6, 1999.

Friedman JM, Birch P. An association between optic glioma and other tumours of the central nervous system in neurofibromatosis type 1. *Neuropediatrics*. 1997 Apr;28(2):131-2.

Rasmussen SA, Friedman JM. NF 1 Gene and neurofibromatosis type 1. *Am J Epidemiol* 151: 33-40, 2000.

Szudek J, Birch P, Friedman JM. Growth charts for children with neurofibromatosis 1 (NF1) *J Med Genet* 92:224-228, 2000.

Papers accepted for publication in peer-reviewed scientific journals

Hamilton SJ, Friedman JM. Insights into the pathogenesis of neurofibromatosis 1 vasculopathy. [In Press: *Clin Genet*]

Szudek J, Birch P, Friedman JM. Analysis of growth in neurofibromatosis 1 (NF1). [In Press: *J Med Genet*]

Szudek J, Birch P, Riccardi VM, Evans DG, Friedman JM. Associations between clinical features of neurofibromatosis 1 (NF1). [In Press: *Genet Epidemiol*]

Szudek J, Evans DG, Friedman JM. Logistic regressive models of clinical features in neurofibromatosis 1 (NF1). [In Press: *J Med Genet]*

Papers submitted for publication

Szudek J, Friedman JM. Unidentified bright objects on MRI associated with cardinal clinical features in neurofibromatosis 1 (NF1). [Submitted]

Platform Presentations at National or International Meetings

DeBella K, Szudek J, Friedman JM. Use of the NIH criteria for diagnosis of NF1 in children. Oral presentation: National Neurofibromatosis Foundation Clinical Conference, October 1998.

DeBella K, Poskitt K, Szudek J, Friedman JM. Use of unidentified bright objects on MRI for diagnosis of neurofibromatosis 1 in children. Oral presentation: American Society of Human Genetics, October 1999. Published abstract: *Am J Hum Genet* 65(3) A36 Supplement, 1999

Palmer C, Joe H, Szudek J, Riccardi VM, Friedman JM. The development of cutaneous neurofibromas is influenced by familial and local factors in patients with neurofibromatosis 1 (NF1). Oral presentation: National Neurofibromatosis Foundation Clinical Conference, October 2000.

Rasmussen SA, Yang QH, Friedman JM. Mortality associated with neurofibromatosis 1 in the United States from 1983 to 1995: an analysis using data from death certificates. Oral presentation: American Society of Human Genetics, October 1999. Published abstract: *Am J Hum Genet* 65(3) A49 Supplement, 1999.

Szudek J, Birch PH, Friedman JM. Height and head circumference in patients with neurofibromatosis type 1. Oral presentation: National Neurofibromatosis Foundation Clinical Conference, October 1998.

Szudek J, Joe H, Friedman JM. Logistic regressive models of neurofibromatosis 1 (NF1) features. Oral presentation: American Society of Human Genetics, October 1999. Published abstract: *Am J Hum Genet* 65(3) A36 Supplement, 1999.

Szudek J, Joe H , Friedman JM. Familial aggregation of neurofibromatosis 1 (NF1) clinical features.. Oral presentation: National Neurofibromatosis Foundation Clinical Conference, October 2000.

Szudek J, Riccardi VM, Friedman JM. Associations of clinical features in children with neurofibromatosis type 1. Oral presentation: National Neurofibromatosis Foundation Clinical Conference, October 1997.

Scientific Poster Presentations at National or International Meetings

DeBella K, Szudek J, Friedman JM. Use of the NIH criteria for diagnosis of NF1 in children. Poster presentation: American Society of Human Genetics, October 1998. Published abstract: *Am J Hum Genet* 63(4) A101 Supplement, 1998.

Palmer C, Szudek J, Joe H, Riccardi VM, Friedman JM. The development of cutaneous neurofibromas is influenced by familial and local factors in patients with neurofibromatosis 1 (NF1).Published abstract: *Am J Hum Genet* 67(Suppl. 2):132, 2000.

Szudek J, Riccardi VM, Friedman JM. Associations of clinical features in children with neurofibromatosis type 1. Poster presentation: American Society of Human Genetics, October 1997. Published abstract: *Am J Hum Genet* 61(4) A115, 1997.

Szudek J, Birch PH, Friedman JM. Height and head circumference in patients with neurofibromatosis type 1. Poster presentation: National Neurofibromatosis Foundation Clinical Conference, October 1998. Published abstract: *Am J Hum Genet* 63(4) A122, 1998.

Szudek J, Joe H , Friedman JM. Familial aggregation of neurofibromatosis 1 (NF1) clinical features. Published abstract: *Am J Hum Genet* 67(Suppl. 2):211, 2000.

Woods RR, Joe H, Evans DGR, Baser ME, Friedman JM. Extension of the Two-hit Hypothesis in neurofibromatosis 2 (NF2): effects of the mutant allele and prediction of the age of onset for both vestibular schwannomas. Published abstract: *Am J Hum Genet* 67(Suppl. 2):107, 2000.

Conclusions

During the project's first year, we used association studies to identify subgroups of NF1 patients in which at least some of the phenotypic variability appears to result from genetic factors, and we set up molecular techniques to identify *NF1* gene mutations.

During the second year, statistical methods, including logistic regression, generalized estimating equations and multivariate normal models were used to analyse associations while controlling for the confounding affect of age. In this year, proof of principal was begun for allele-phenotype associations using the "deletion phenotype" as a model.

In the third year, we began to collect blood samples and identify patients who would be appropriate to study for allele-phenotype correlations. The strategic screening process was modified to include processing of RNA to produce cDNA. We also used more sophisticated statistical methods, including multivariate probit modelling, in order to investigate the sources of phenotypic variability for certain familial features of NF1. We were able to differentiate between features which appeared to be influenced by the *NF1* allele from those influenced by the normal *NF1* allele, and by modifying genes unlinked to *NF1*.

In the fourth and final year, we shall finish the allele-phenotype correlational studies and will continue to examine other possible effects such as the epigenetic influences of imprinting.

These studies are providing considerable insight into the causes of clinical variability of NF1. An understanding of the reasons for this clinical variability is a necessary prerequisite to understanding the genetics of NF1 and is central to genetic counselling as well as to development of new approaches to therapy.

Appendix

1. Pre-print:

Szudek J, Birch P, Riccardi VM, Evans DG, Friedman JM. Associations between clinical features of neurofibromatosis 1 (NF1). [In Press: *Genet Epidemiol*]

2. Submitted for Publication:

Szudek J, Evans DG, Friedman JM. Logistic regressive models of clinical features in neurofibromatosis 1 (NF1). [In Press: *J Med Genet]*

3. Abstracts

Palmer C, Szudek J, Joe H, Riccardi VM, Friedman JM: The development of cutaneous neurofibromas is influenced by familial and local factors in patients with neurofibromatosis 1 (NF1).Published abstract: *Am J Hum Genet* 67(Suppl. 2):132, 2000.

Szudek J, Joe H , Friedman JM. Familial aggregation of neurofibromatosis 1 (NF1) clinical features. Published abstract: *Am J Hum Genet* 67(Suppl. 2):211, 2000.

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Associations of Clinical Features in Neurofibromatosis 1 (NF1)

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Neurofibromatosis 1 (NF1), an autosomal dominant disease, exhibits extreme clinical variability. This variability greatly increases the burden for affected families and impairs our ability to understand the pathogenesis of NF1. Recognition of heterogeneity within a disease may provide important pathogenic insights, therefore we tested clinical data from three large sets of NF1 patients for evidence that certain common features are more likely to occur in some NF1 patients than in others. Clinical information on 4,402 patients with NF1 was obtained from three independent databases. We examined associations between pairs of clinical features in individual affected probands. We also examined associations between the occurrence of individual features in affected relatives. Associations were summarized as odds ratios with 95% confidence intervals. We found associations between several pairs of features in affected probands: intertriginous freckling and Lisch nodules, discrete neurofibromas and plexiform neurofibromas, discrete neurofibromas and Lisch nodules, plexiform neurofibromas and scoliosis, learning disability or mental retardation and seizures. We also found associations between the occurrence of Lisch nodules, macrocephaly, short stature, and learning disability or mental retardation as individual features in parents and children with NF1.

Our observations suggest that, contrary to established belief, some NF1 patients are more likely than others to develop particular manifestations of the disease. Genetic factors appear to determine the development of particular phenotypic features. Genet. Epidemiol. 19:00–00, 2000. © 2000 Wiley-Liss, Inc.

Key words: database; phenotype; proband; familial

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INTRODUCTION

Neurofibromatosis 1 (NF1) is a progressive autosomal dominant disorder affecting approximately 1 in 3,000 people [Crowe et al., 1956; Huson et al., 1989; Littler and Morton, 1990]. Its most frequent features include café-au-lait macules, Lisch nodules, discrete and plexiform neurofibromas, and learning disabilities. Although NF1 has been recognized clinically for more than 100 years [von Recklinghausen, 1882], its natural history is not completely characterized and the pathogenesis is poorly understood. The disease is fully penetrant, but expressivity is variable [Riccardi, 1992]. This variability confounds clinical management and genetic counseling.

The *NF1* locus, identified and sequenced 9 years ago, is the second largest human gene known [Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990]. Owing to its large size, the lack of clustering of mutation sites and the fact that recurrent mutations are uncommon, molecular genetic testing is not routinely used [Gutmann et al., 1997]. NF1 remains a clinical diagnosis based on the presence of characteristic physical signs or an affected first-degree relative [NIH, 1988; Gutmann et al., 1997]. *NF1* gene mutations have been reported in fewer than 300 patients [NNFF, 1999].

Manifestations of NF1 vary at different times in an individual's life [Moritz and Sneider, 1962; Fitzpatrick et al., 1983; Knight and Reiner, 1983; Riccardi, 1992; Dugoff and Sujanksy, 1996]. Substantial variability also exists among affected members of a single family [Crowe et al., 1956; Zoller et al., 1995]. Nevertheless, there is evidence that related individuals with NF1 are more similar to each other than to unrelated affected individuals. Easton et al. [1993] 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. There are also families in which an unusual phenotype imparted by an *NF1* mutation appears to "breed true." For example, in families with the Watson syndrome variant of NF1, affected relatives all have features of Watson syndrome rather than typical NF1 [Upadhyaya et al., 1990; Allanson et al., 1991]. In other families, mutations of the *NF1* locus appear to be expressed consistently as multiple café-au-lait spots without other manifestations of NF1 [Abeliovich et al., 1995] or with spinal neurofibromas in multiple generations [Pulst et al., 1991; Poyhonen et al., 1997; Ars et al., 1998].

Recognition of clinical heterogeneity within a disease may provide important pathogenic insights. For example, understanding that NF1 and NF2 are different diseases [Riccardi, 1982] was a seminal contribution. To determine whether clinical heterogeneity exists within NF1 itself, we tested three large clinical datasets for associations between pairs of clinical features in probands. We also tested for genetic determinants of clinical variability by looking for associations of individual clinical features between parents and children with NF1. We found consistent associations among the occurrence of different clinical features in individual patients and between the occurrence of the same feature in relatives. Our observations complement those of Easton et al. [1993] and suggest that, contrary to traditional belief [Bernhart and Halperin, 1990; Riccardi, 1992], some NF1 patients are more likely than others to develop particular manifestations of the disease.

SUBJECTS AND METHODS

Patients and Data Description

All patients included in this analysis were diagnosed with NF1 according to established clinical criteria [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, 1997] contained descriptions of 2,509 NF1 probands, 211 affected parents, and 289 of their affected children. Of the NF1 cases 83% 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. Of the cases 72% are Caucasian, 14% Hispanic, 13% African American, and 1% Asian. The Manchester NF1 database (MANF1) [McGaughran et al., 1999] includes clinical information on 270 probands, 94 affected parents, and 140 of their affected children. Of the cases 92% are Caucasian, 4% Indian, 2% black, 1% Bangladeshi, and 1% Pakistani. There is no overlap among the patients included in these three databases. Specific *NF1* mutations have been identified by molecular analysis in <1% of these patients.

Statistical Analysis

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). Table I summarizes 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 ≥2 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 ≥2 standard deviations above the age- and gender- 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 magnetic resonance imaging or computed tomography examination. Only patients who had definite presence or absence of a feature were considered in comparisons involving that feature.

Pair-wise combinations of the presence or absence of each feature were analyzed in probands by 2×2 tables using SAS [SAS Institute, 1996]. The prevalence of many features of NF1 increases with age [Riccardi, 1992; Cnossen et al., 1998]. Two

TABLE I. 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 in our studies)

	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)	86.0	(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.

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 sparse. The method of Mantel and Haenszel [1959] was used to estimate the odds ratio with 95% confidence intervals over the age and gender strata. We performed the analyses in three independent datasets (NFDB, NFID, and MANF1) because we expected to observe many associations that 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 owing to chance alone. The Breslow-Day method [SAS Institute, 1996] was used to test each triad of odds ratios for homogeneity between the three databases. Triads with P-values >0.05 were considered homogeneous. The method of Mantel and Haenszel [1959] was used to estimate the summary odds ratio with 95% confidence intervals for all three databases together.

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, we limited our analysis to parents and children, who usually differ in age by at least 20 years. For each of the 12 features, a 2×2 table was used to compare the frequency of a given feature in NF1 children of NF1 parents 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 Institute, 1996] was used to test for homogeneity, and the Mantel-Haenszel method [1959] was used to estimate summary odds ratios.

RESULTS

Associations Between Features in Individual NF1 Probands

Pair-wise associations between each of the 12 clinical features were tested in 2,509 NF1 probands from the NFDB, 774 NF1 probands from the NFID, and 270 NF1 probands from the MANF1 database (Table II). 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 three nominally significant inverse associations. In the NFID, which contains fewer than one third 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 three were negative. In the MANF1, which is approximately one ninth as large as the NFDB, an odds ratio of 1.0 was excluded from the 95% confidence limits for five of 55 associations tested. All these were positive. Odds ratios could not be calculated in the remaining 11 associations owing to blank cells in the contingency tables. Overall, six of 66 tested associations between pairs of features are statistically significant and in the same direction in at least two of the databases (Table II). Four of these six associations are statistically homogeneous between the three databases. One statistically significant inverse association was observed in at least two independent databases. The associations are in Table II as odds ratios for each database and as summary odds ratios for all three databases together.

Associations of Features Between Affected Parents and Children

Table III summarizes 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 owing to blank cells in contingency tables. A summary odds ratio of one was excluded from the 95% confidence limits in four of the 12 associations between parents and children. Three of these four associations are statistically homogeneous between the three databases. No significant negative associations were observed.

DISCUSSION

Associations Between Features in Individual NF1 Probands

The large number of cases in these three databases enables us to find significant associations between several common features of NF1 (Table II). The concordance between the findings in the three independent databases is remarkable. Approximately 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 NFDB, NFID, and MANF1. The reproducibility of our results suggests that these associations are probably not owing to chance alone.

The positive associations observed may reflect shared pathogenic mechanisms underlying the associated features. For example, NF1 probands with seizures may be

TABLE II. Odds Ratios with 95% Confidence Limits for Associations of Features Among Age and Gender-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 II are blank.
NFs, neurofibromas; LD/MR, learning disability or mental retardation.

TABLE III. Odds Ratio 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 (3.5–225)	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 (1.9–595)	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 III are blank.
NFs, neurofibromas; LD/MR, learning disability or mental retardation.

more likely also to have learning disabilities or mental retardation than patients without seizures (Table II) 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 II) 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 loss or mutation of the normal *NF1* allele in at least some cases [Serra et al., 1997; Sawada et al., 1996]. 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 we observed is much stronger in younger than in older NF1 patients. The odds ratio was 6.9 among patients younger than 5 years old, 3.1 among those 5 to 9, but only 1.3 among those older than 40. This raises the interesting possibility that NF1 patients with plexiform neurofibromas develop discrete neurofibromas earlier than patients without plexiform lesions.

The associations between Lisch nodules and both discrete neurofibromas and intertriginous freckling (Table II) were previously reported [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 et al., 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, such as the sympathetic ganglia, thyroid C-cells, and parathyroids, are rarely involved in NF1. Moreover, many features of NF1, such as learning disabilities, dysplastic scoliosis, and tibial pseudarthrosis, do not appear to be abnormalities of neural-crest derived tissues.

Although plexiform neurofibromas growing near the spine can cause abnormal

curvature and result in scoliosis, the association we observed between plexiform neurofibromas and scoliosis does not lose significance when patients with 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 common adolescent scoliosis [Riccardi, 1992]. The association we observed involves only early-onset scoliosis. The pathogenic basis for this association and for the association we observed between the absence of pseudarthrosis or bowing and discrete neurofibromas 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 II). Such pairwise associations are not strong enough to be useful clinically for predictive classification of patients. Nevertheless, our 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 generally held view that any NF1 patient may develop any manifestation of the disease [Bernhart and Halperin, 1990; Riccardi, 1992]. Most of the associations we observed have never been noted before. Their identification is an important step toward understanding the pathogenesis of NF1.

Associations of Features Between Affected Parents and Children

Our observations in probands suggest that shared pathogenic mechanisms underlie several common features of NF1. If genetic factors influence these pathogenic mechanisms, one would expect familial aggregation of such features to occur. Therefore, we 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. Owing to this age difference, we expect the odds ratios from parent-child comparisons to yield conservative estimates of intra-familial associations. Consequently, we limited our analysis to affected parents and children.

Several strong associations were found by comparing the presence or absence of the 12 features between affected parents and children (Table III). The summary odds ratios for Lisch nodules, optic glioma, macrocephaly, and short stature were significant and homogeneous among the three databases. The summary odds ratio for learning disability or mental retardation was significant but was not homogeneous between the three databases. This may be owing to differences among centers in how the feature is diagnosed. These associations are probably not owing to ascertainment bias. All subjects were assessed in specialized 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.

No negative associations were found between affected parents and children. This is consistent with our hypothesis that affected relatives have a more similar NF1

phenotype than unrelated people. The absence of negative associations also supports the statistical validity of our observations. One would expect to observe negative, as well as positive, associations by chance.

We 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 intrafamilial 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 not examined here. Unlike our study, the results of Easton et al. rely heavily on data from six pairs of monozygotic twins. Nevertheless, the studies are both consistent with genetic factors influencing the phenotypic expression of *NF1* mutations in patients with NF1.

The strong 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 large 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 variants of the normal *NF1* allele and "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. Our findings complement those of Easton et al. [1993] and are consistent with their hypothesis hat 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]. Functional variants of these proteins might also influence the NF1 phenotype.

Our studies demonstrate that, although the NF1 phenotype is highly variable, some patients are more likely than others to develop certain disease features. In addition, we show that genetic factors may determine 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 our investigations provide hope that some serious complications of NF1 can be predicted or prevented.

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LOGISTIC REGRESSIVE MODELS OF CLINICAL FEATURES IN NEUROFIBROMATOSIS 1 (NF1)

Running Title: Logistic Regression in NF1

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SUMMARY

Neurofibromatosis 1 (NF1) is a common, fully penetrant, autosomal dominant disease. The clinical course is generally progressive but highly variable, and the pathogenesis is poorly understood. A better understanding of this variability may shed light on its pathogenesis.

We studied interactions among 13 of the most common or important NF1 clinical features in data on 2797 NF1 probands (divided into separate developmental and validation subsets) and 511 of their affected relatives from the NNFF International Database and on 441 NF1 patients from a population-based registry in north-west England. We developed logistic regressive models for each of the 13 features using the developmental sample and attempted to validate these models in the other 3 samples. Age and gender were included as covariates in all models.

Models were successfully developed and validated for 10 of the 13 features analyzed. The results 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. In addition, 3-way interactions among café-au-lait spots, intertriginous freckling and subcutaneous neurofibromas suggest that the first two groups are not independent.

Clinical features within a group may share pathogenic mechanisms that differ, at least in part, from those underlying features in other groups. The results suggest a variety of familial and molecular investigations into the pathogenesis of NF1.

KEY WORDS

phenotype, expressivity, database, pathogenesis

INTRODUCTION

Neurofibromatosis 1 (NF1) expressivity is tremendously variable [Friedman et al. 1999], 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* gene and in families with NF1 variants such as Watson syndrome [Upadhyaya et al. 1990; Allanson et al. 1991]. In these cases, particular genotypes result in specific constellations of clinical features.

In a previous study, we demonstrated several associations between pair-wise combinations of clinical features among age-stratified probands with NF1 [Szudek et al. in press]. 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 we analyzed age in 10-year strata, there still may have been considerable age-related variability, especially among the youngest patients. In this study, we have extended our 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. Our findings clarify and refine the associations among clinical features in NF1 patients and provide further clues to the pathogenesis of these features.

SUBJECTS AND METHODS

This study involved analysis of four separate clinical samples of patients with NF1 – the developmental, validation, relative, and population-based Manchester samples, as described below. Logistic regressive models were built from an initial series of univariate 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.

Subjects

Subjects were obtained from two large clinical databases: the National Neurofibromatosis Foundation International Database (NFDB) and the Manchester NF1 database (MANF1). All patients included in this analysis were diagnosed with NF1 according to established clinical criteria [NIH 1988; Gutmann et al. 1997]. The NFDB includes 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. Subject age at exam ranged from birth to 89 years. All information was collected and recorded on each patient using a standard procedure [Friedman and Birch 1997]. The data were audited for quality and consistency by the NFDB administrator. 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]. Probands and affected relatives were studied as a single group in the MANF1 because there were not enough cases to permit separate analysis. 92% of the cases are Caucasian, 4% Indian, 2% Black, 1% Bangladeshi and 1% Pakistani. There is no overlap among the patients included in the NFDB and MANF1 databases.

Clinical Features

We 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.

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 2 or more standard deviations below the age- and sex-matched population mean. Subjects with pseudarthrosis, early or delayed puberty, scoliosis, vertebral dysplasia, or spinal compression were excluded from analyses involving height because these features may alter what the height would otherwise be. Macrocephaly was coded as "present" if the subject's head circumference was 2 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 because these features may alter the head circumference. Standard population norms for height and head circumference by age were obtained from the National Center for Health Statistics and the Fels Institute [Hamill et al. 1977]. Lisch nodules were diagnosed or excluded by a slit lamp examination; individuals who did not have a slit lamp examination were coded as "unknown". 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". Patients coded as "unknown" for a particular feature were not considered in models involving that feature.

Statistical Models

Thirteen separate logistic regression models were built, with the logit of each of the 13 NF1 features analysed set as the response variable (Y) in a different model. 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 univariate model was constructed using age as the only covariate:

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

where $p(1|x)$ is $Pr(Y=1|$ covariates $x)$.

Maximum likelihood techniques were used to generate parameter estimates [SAS Institute 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 *AGETRF* approaches α as *AGE* gets larger. This function approximates the frequency-by-age curves of the NF1 features considered in this study [DeBella et al. 2000]. It was necessary to use this transformation of AGE to maintain linearity of the logit for all outcome variables in this study.

A series of bivariate 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 (Y) 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 $p<0.2$ were included as explanatory variables (x_i's) in multivariate 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 multivariate 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,

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

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 asses how well the parameter estimates from the developmental sample fit the validation, affected relative, and MANF1 samples. 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, affected relative, and MANF1 samples, to allow more detailed comparison.

Interpretation

Logistic regressive models have a straightforward interpretation in terms of odds-ratios. The strength of interaction between the response variable (Y) and an explanatory variable (x_1) in a univariate 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 bivariate model is measured by β_1, β_2, and δ_1. Subjects with variables x_1 and x_2 present are $\exp(\beta_1+\beta_2+\delta_1 x_1x_2)$ times more likely to also have the response feature. Odds ratios with 95% confidence intervals that excluded 1.0 were considered unlikely to

be due to chance alone.

RESULTS

A multivariate logistic regressive model was generated for each of 13 different NF1 clinical features, using age, 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 – a second, "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 also had an adequate fit in the validation, affected relative and MANF1 samples: intertriginous freckling, subcutaneous neurofibromas, plexiform neurofibromas, optic glioma, pseudarthrosis, macrocephaly, and other neoplasms (Table I). Models for café-au-lait spots, cutaneous neurofibromas, Lisch nodules, seizures, scoliosis and short stature had an inadequate fit in at least one of the samples.

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 II). 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 II are blank.

Some of the parameters from models that had an adequate fit in all four samples were not consistent when generated independently in each sample. In the plexiform 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 I and are summarized as odds ratios with 95% confidence intervals in Table III. For example, intertriginous freckling was found to be 20% more common in subjects with café-au-lait spots, 40% less common in those with subcutaneous neurofibromas, and 30% more common 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.

DISCUSSION

The models we 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 specialized 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 specialized clinics, but their severity is not as biased as that of the probands [Friedman and Birch 1997]. 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. We 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. Its ascertainment is near 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 specialized clinic biases. Instead these models probably reflect trends that exist in the NF1 population at large.

Features such as optic glioma, seizures, pseudarthrosis naturally fall into a binary 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 avoid uncertainties in the collection of quantitative data from many different NFDB contributing centres and to permit comparison of NFDB models in the MANF1.

Many of the associations in Table III do not have 95% confidence intervals that exclude 1.0. However, several of these models include three-way interactions (Table II), 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 is often extended to $p<0.2$.

Associations limited to freckling, Lisch nodules, and plexiform, cutaneous and subcutaneous neurofibromas, have been previously reported as pair-wise associations of weak magnitude [Szudek et al. in press]. 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 study shows that most of these associations not only persist when controlling for other common NF1 features, but increase slightly in strength when the presence of multiple features is considered. The presence of café-au-lait spots, subcutaneous neurofibromas as well as Lisch nodules, make freckling 3.7 (95% C.I.=1.8-7.4) times more likely. Furthermore, this study shows that these pair-wise associations exist side-by-side. For example, cutaneous and subcutaneous neurofibromas are both significantly associated with plexiform neurofibromas (Table III).

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 plexiforms and macrocephaly, as well as neoplasms, are present.

Easton et al. [1993] 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. Our study was of individual NF1 patients, not of familial associations, but both studies are consistent with genetic factors contributing to the development of several common NF1 features.

Many of the associations we observed were non-reciprocal – only one of a pair of features appears in the others' model. This suggests that the two features were not of primary importance in accounting for each other's status. This might occur, for example, if both features result from a common pathogenic factor that was not itself included in the models. The reciprocal associations we observed are consistent with the existence of three groups of features among the 13 features studied (figure 1). In general, features were considered to be grouped if each feature appeared as an explanatory variable with a positive parameter estimate in each of the other group members' models. Fundamental pathogenic 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.

Café-au-lait spots, intertriginous freckles and Lisch nodules are all derived from cells of melanocytic origin [Weston et al. 1981; Perry and Font 1982]. Café-au-lait spots contain melanosomes with giant pigment particles. Intertriginous freckles derive from a

genetic pathway that has nothing to do with 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.

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 [Serra et al. 1997; Sawada et al. 1996]. The negative 3-way interaction terms in two of the three models suggest that associations involving neurofibromas are not independent.

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 I). 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. Café-au-lait spots, intertriginous freckles and subcutaneous neurofibromas all involve cells derived from the embryonic neural crest [Weston et al. 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, such as the

sympathetic ganglia, thyroid C-cells, and parathyroids, are rarely involved in NF1. Moreover, many features of NF1, such as learning disabilities, dysplastic scoliosis, and tibial pseudarthrosis, do not appear to be abnormalities of neural-crest derived tissues.

The common thread between optic glioma, other neoplasms and macrocephaly could be glial hyperplasia resulting from haploinsuffiency of neurofibromin. Most of the other neoplasms in our patients 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 [Huson 1994; Riccardi 1992]. Gutmann et al. [1999] have directly demonstrated an effect of *NF1* haploinsufficiency on glial cell proliferation.

The pathogenic basis for the association we observed between pseudarthrosis and other neoplasms is not well understood.

While these models are accurate descriptors of feature occurrence, they cannot be used to predict who will get what features. The NFDB data is 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.

Phenotypic studies of affected relatives can determine the importance of familial and genetic factors in the development of these common NF1 features. Family studies on NFDB patients may differentiate between the different familial mechanisms that could be contributing to NF1 expressivity.

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FIGURE LEGEND

Figure 1: Proposed grouping of NF1 features, based on the odds ratios in Table III. Features enclosed by a box or connected by an arrow are important variables in each other's models.

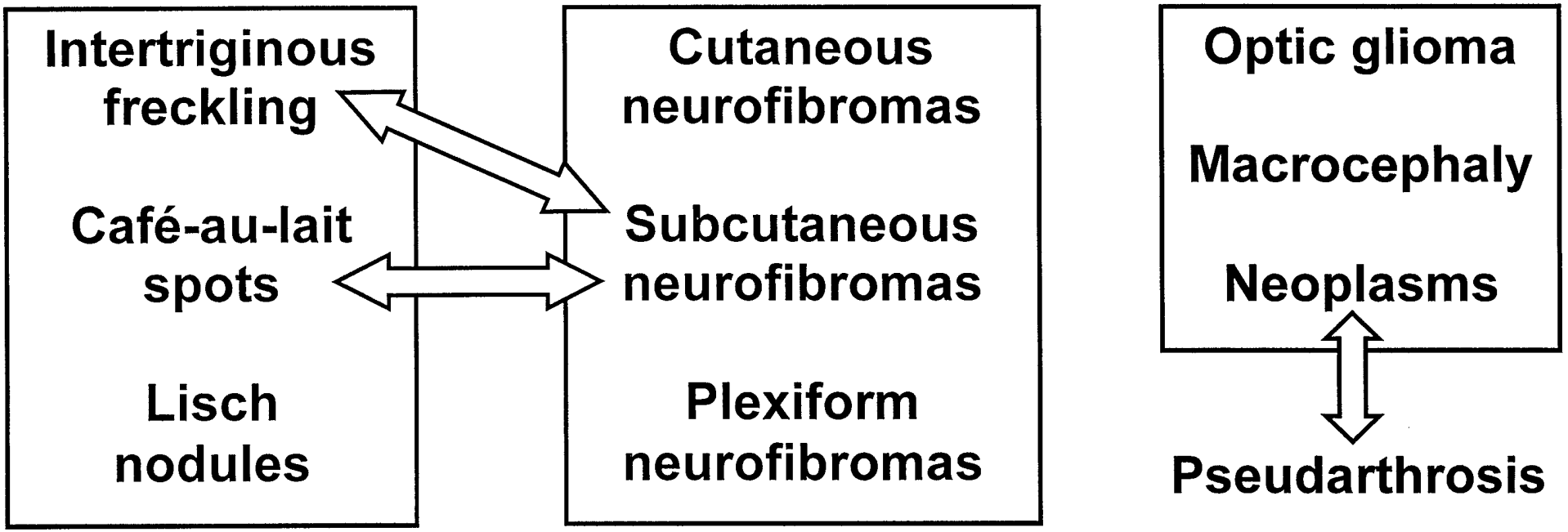
Intertriginous freckling
Café-au-lait spots
Lisch nodules
Cutaneous neurofibromas
Subcutaneous neurofibromas
Plexiform neurofibromas
Optic glioma
Macrocephaly
Neoplasms
Pseudarthrosis

Table I: Summary of goodness-of-fit and sample size for logistic regressive models of NF1 clinical features. Parameters (β's) were estimated in the developmental subsample and their fit was compared to three other subsamples. Explanatory features separated by asterisks represent interaction variables.

Output Feature	Explanatory	β	Development	Validation	Relatives	Manchester
Café-au-lait spots (CLS)	Freckling SNF Lisch nodules Freckling*SNF	0.25 -0.63 0.38 1.12	p=0.59 n=987	p=0.88 n=985	p<0.01 n=350	p=0.46 n=180
Freckling	CLS SNF Lisch nodules CLS*SNF	0.16 -0.46 0.29 1.31	p=0.88 n=987	p=0.23 n=985	p=0.20 n=350	p=0.41 n=180
Cutaneous neurofibromas (CNF)	SNF Plexiform Pseudarthrosis	0.48 0.67 -0.61	p=0.59 n=1372	p=0.12 n=1352	p=0.13 n=492	p<0.01 n=281
Subcutaneous neurofibromas (SNF)	CLS CNF Plexiform CNF*Plexiform	0.30 0.69 0.87 -0.63	p=0.16 n=1358	p=0.06 n=1331	p=0.07 n=487	p=0.47 n=294
Plexiform neurofibromas	CNF SNF Scoliosis Neoplasm SNF*CNF	0.85 1.13 0.62 -0.39 -0.71	p=0.75 n=1196	p=0.19 n=1221	p=0.10 n=416	p=0.06 n=250
Lisch nodules	CLS CNF Neoplasm CLS*CNF	0.44 0.67 1.74 -0.38	p=0.74 n=969	p=0.63 n=971	p=0.50 n=348	p=0.01 n=172
Optic glioma	Plexiform Macrocephaly Neoplasm	0.66 0.48 1.97	p=0.50 n=313	p=0.74 n=328	p=0.50 n=87	p=0.09 n=172
Seizures	SNF Neoplasm Male gender SNF*Male Neo*Male	0.10 2.09 0.20 0.93 -2.05	p=0.47 n=1300	p<0.01 n=1306	p=0.22 n=473	p=0.10 n=257
Pseudarthrosis	Freckling CNF Neoplasm Male gender	-0.80 -0.54 0.79 0.54	p=0.62 n=1270	p=0.10 n=1285	p=0.38 n=461	p=0.38 n=258
Scoliosis	CNF Plexiform	-0.75 0.71	p=0.04 n=1289	p=0.03 n=1289	p<0.01 n=447	p=0.71 n=322
Macrocephaly	Lisch nodules Optic glioma Short stature Neoplasm	0.45 1.05 -1.43 -2.11	p=0.79 n=170	p=0.41 n=190	p=0.39 n=57	p=0.32 n=79
Short stature	CLS CNF Macrocephaly	-0.63 0.40 -1.21	p=0.57 n=620	p=0.07 n=626	p=0.01 n=261	p<0.01 n=171
Other neoplasms	Lisch nodules Optic glioma Pseudarthrosis	0.94 1.93 1.76	p=0.93 n=411	p=0.07 n=439	p=0.92 n=117	p=0.39 n=141

Table II: 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.

Output Feature	Explanatory Feature	Development	Validation	Relatives	Manchester
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
	Plexiform	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
	Plexiform	0.87	0.74	1.01	0.73
	CNF*Plexiform	-0.63	-0.58	-0.83	-0.10
Plexiform neurofibromas	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
Optic glioma	Plexiform	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		
	Neo*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
	Plexiform	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
Other neoplasms	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 II are blank.

Table III: Summary of consistent associations from validated logistic regressive models of NF1 clinical features.

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)
	Plexiform	2.4 (1.6-3.6)
	All three	*3.4 (2.1-5.7)*
Plexiform neurofibromas	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	Plexiform	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)*
Other neoplasms	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)*

The Development of Cutaneous Neurofibromas is Influenced by Familial and Local Factors in Patients with Neurofibromatosis 1 (NF1).

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NF1 is an autosomal dominant condition affecting 1 in 3000 individuals. Its defining feature is the neurofibroma: a complex benign tumor arising from peripheral nerve sheaths. The number of cutaneous neurofibromas in NF1 patients increases with age and is highly variable; the cause of this variability is unknown. We tested the hypothesis that development of these lesions may be influenced by local and familial factors.

The presence or absence of 1 or more cafe au lait spots, 1 or more cutaneous neurofibromas, and 1 or more diffuse plexiform neurofibromas was recorded for each of ten divisions of the body surface in 768 NF1 patients, including 117 affected individuals in 52 families. We used a random effects model to obtain the maximum likelihood estimate and confidence interval of intrafamilial correlations in the number of body divisions affected with 1 or more cutaneous neurofibromas, while controlling for age. The correlation amongst first-degree relatives was r=0.30 (95% CI=0.078,0.52), in agreement with previous studies.

We used a Mantel-Haenszel test, stratified simultaneously by body division and number of body divisions with 1 or more cutaneous neurofibromas, to examine associations in the presence of diffuse plexiform neurofibromas and cutaneous neurofibromas in individual NF1 patients (n=630). Divisions that include a diffuse plexiform neurofibroma are twice as likely to have 1 or more cutaneous neurofibromas as well (summary odds ratio=2.02; 95% CI=1.28, 2.77). Odds ratios were not homogeneous across body divisions. No significant association was observed between the presence of cafe au lait spots and cutaneous neurofibromas in a body division in NF1 patients (n=584).

We conclude that the occurrence of cutaneous neurofibromas in NF1 patients is influenced by familial factors as well as by local factors.

Familial Aggregation of Neurofibromatosis 1 (NF1) Clinical Features

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The gene responsible for NF1 was cloned over ten years ago, but the relationship of genetic factors at the *NF1* locus or other loci to development of specific disease features is poorly understood. We have shown previously that NF1 features do not occur independently in individuals. We now extend the analysis to include familial aggregation of NF1 features among different classes of affected relatives.

The National Neurofibromatosis Foundation International Database includes extensive cross-sectional information on 320 families with 2 or more members affected with NF1 according to the NIH Diagnostic Criteria. The 786 NF1 patients in these families include 223 sib-sib, 290 parent-child and 70 second degree relative pairs. For this analysis, we selected 13 of the most common or important clinical features of NF1: café-au-lait spots, intertriginous freckling, cutaneous, subcutaneous and plexiform neurofibromas, Lisch nodules, seizures, pseudarthrosis, scoliosis, macrocephaly, short stature, optic gliomas, and other neoplasms.

We used multivariate logistic regression to measure aggregation of risk among relatives while adjusting for individual covariates. The logit of each of the 13 features was set as the output variable in a different regression model. Two separate regressions were simultaneously applied in each model. The first accounted for associated features and covariates such as age and gender. Associated clinical features were treated as binary variables with relationships established by our previous studies of individual NF1 patients. Age was controlled as a continuous covariate. The other regression was used to assess familial aggregation of the response variable between three different classes of affected relatives: sibs, parent-child pairs and second-degree relatives

All of the features except plexiform neurofibromas, optic glioma, seizures and scoliosis appear to be familial. Among the familial features, odds ratios between sibs ranged from 4.4 (95% CI 2.1-6.7) for café-au-lait spots to 15.2 (95% CI 4.0-26.5) for cutaneous neurofibromas. Odds ratios between parents and children ranged from 2.3 (95% CI 0.4-4.3) for freckling to 6.0 (95% CI 1.1-10.9) for macrocephaly. Odds ratios between second degree relatives ranged from 1.0 (95% CI 0.5-1.5) for freckling to 9.5 (95% CI 4.2-14.8) for cutaneous neurofibromas. Although the confidence intervals were broad three distinct patterns were observed among the point estimates for familial features: 1) Odds ratios for some features are similar for all relationships; 2) Others have higher odds ratios between first degree relatives than between second degree relatives; 3) Some are higher between sibs than between parents and children. These familial patterns suggest that the 1) the mutant *NF1* allele, 2) unlinked modifying genes and 3) the normal *NF1* allele may all be involved in the development of particular clinical features of NF1, but that their relative importance varies for different features.