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13. ABSTRACT *(Maximum 200 words)*

The clinical manifestations of NF1 are extremely variable. The purpose of this project is to characterize the sources of phenotypic variability in NF1 by using a combination of clinical, statistical, epidemiological, and molecular genetic methods. Our studies to date have included analysis of possible associations among all clinical features by simple 2x2 X^2 analysis and more detailed analysis of selected features for the effects of covariates using stratification and log-linear methods. We found that age does not have a uniform effect on each variable and have developed a strategy using various approaches including linear regression to deal with this problem in future analyses.

We have developed a list of possible clinical subtypes of NF1. We are currently analysing several of these subsets by a combination of clinical, genetic, and statistical approaches to determine which are most likely to represent allelic variants of NF1. Our collaborator, Dr. David Viskochil has implemented most of the molecular techniques required for the strategic screening process we shall use for identification of constitutional mutations of *NF1*.

During the second year of the project, we shall begin testing selected phenotypic subgroups of NF1 patients using our molecular case-control studies.

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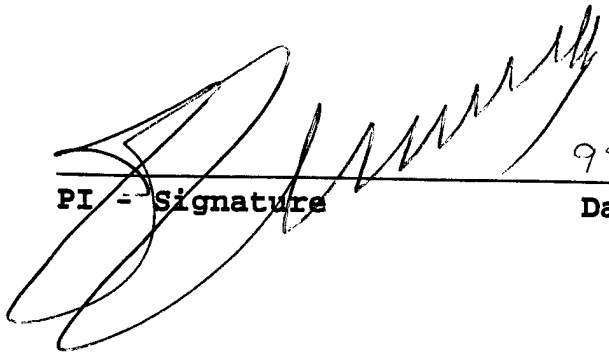

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INTRODUCTION

The natural history of neurofibromatosis type 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 three-year project is to characterise the sources of phenotypic variability in NF1. We are pursuing this goal using a combination of clinical, statistical, epidemiological, and molecular genetic methods.

Our specific objectives are to:

1. identify statistical associations between pairs of clinical features in unrelated NF1 patients in data from the National Neurofibromatosis Foundation International Database (NNFFID) and from the NF Institute database, an independent data set;
2. identify statistical associations between the occurrence of individual clinical features in NF1 probands and their affected family members in data from the NNFFID or from the NF Institute database;
3. derive age-specific risks for particular complications of NF1 in patients with and without certain associated disease manifestations;
4. derive age-specific risks for particular complications of NF1 in the relatives of patients with and without the same complication;
5. determine the contribution of genetic factors (such as allelic differences and modifying genes), non-genetic factors, and chance to the variability of occurrence of serious complications of NF1; and
6. identify allele-phenotype correlations for clinical features for which allelic heterogeneity has been shown to be an important source of clinical variability.

During the first year of this project, our emphasis has been on objectives 1 and 2, and on implementing the molecular genetic screening process required for objective 6. More specifically, our goals were to

- use association studies and other methods to identify subgroups of NF1 patients in which at least some of the phenotypic variability appears to result from genetic factors and
- to set up the full array of molecular techniques necessary for identification of up to 90% of *NF1* gene mutations.

PROGRESS REPORT - ASSOCIATION STUDIES

Goal 1. To identify associations between features of NF1

Task 1: Identify associations of features within probands by screening all possible pair-wise associations;
Task 2: Identify associations of NF1 features between related individuals (e.g. parent -child diads);
Task 3: Examine apparent associations in detail for other covariates or confounding factors using log-linear models .

METHODS – Association Studies

We began our analysis by producing two-by-two tables for the approximately 100 features in the NNFFID (Task 1). This resulted in a total of more than 5000 tables. Our plan was to examine statistically significant associations for covariates such as age, gender and race, then to examine each association for the presence of higher order associations. In order to identify all potential associations, we used *chi-square* calculations with a nominal significance level of p=.05 and odds ratios with 95% confidence intervals that do not overlap 1 to identify potential associations for further study. A more conservative Bonferoni correction for multiple comparisons was not used in order to avoid eliminating small, but potentially important associations.

We examined potential associations for covariates (task 3) using methods appropriate for the covariate: For binary variables such as gender, or categorical variables such as race, or parent of origin, we were able to perform 2 x 2 x n tables (where "n" represents the number of categories of the potential covariate). The same statistical tests of significance were applied as for the 2 x 2 tables. When we examined the effect of age on various associations, we realised that age does not have a uniform effect on each variable (see discussion in Results and Problems and Proposed Solutions, below).

In order to explore this problem more fully, we graphed the prevalence of all clinical features of NF1 among patients in the NNFFID by age: The frequency of each feature was plotted in one-year intervals throughout childhood and in 2 ½ to 10 year increments in adulthood, depending on the overall prevalence of the feature. (For the uncommon features, a wider age interval was used to have sufficient cases per category). All patients that met the NIH criteria for NF1 were potentially included in each plot and unknowns were excluded. Samples of these graphs appear in appendix A, figures 1 to 6).

We are now beginning to analyze more complex multi-way associations of clinical features in individual patients and their families. For many of these associations we are using log-linear modelling [Beaty et al, 1991] which takes into account the interaction

between two variables in the presence of a third, or higher order interaction.

Our second approach for analysis of combinations of features that vary with age is to use logistic regressive modelling, which we originally planned to apply to just a subset of traits later in the project. (See further discussion of this issue in "Problems and Proposed Solutions, below).

We are also studying several subsets of NF1 patients in detail to determine if they comprise appropriate groups in which to pursue genotype-phenotype correlations. These patients have unusual complications of NF1 that might be indicative of distinctive pathogenic mechanisms. Our working hypothesis is that variations in pathogenesis may reflect variations in the specific mutations present in the NF1 gene.

We are selecting these subsets based on our own observations of unusual features in the database or on evidence of possible subtypes reported in the NF1 literature. We are trying to define potential subsets as clearly as possible clinically by means of detailed analysis of patients in NNFFID who appear to have the particular combination of features under consideration. This sometimes involves contacting the original database contributor for more information about the patient and family members. An example of this is discussed in the Results section, under the subheading "Cardiovascular anomalies." Possible phenotypic subsets are also discussed in the Results section, below.

We have initiated our collaborations with Doctors Riccardi, Viskochil, and Khoury. We are working with Dr. Riccardi to develop a fully computerized database of the detailed clinical and genetic information that he has collected on more than 1000 NF1 patients. We have made paper copies of these records and have transferred the data into an electronic format.

We have recently gained access to an additional database of over 500 NF1 patients from Dr. Gareth Evans. This is a population-based database that represents approximately 70% ascertainment of NF1 patients in Northwest England. Dr. Evans' database is, therefore, likely to be more representative of the NF1 population as a whole than are the NNFFID and NFI databases, which include only patients that were referred to specialized centres. Dr. Evans' data also contain more complete families and more adult cases than either of the other two databases.

RESULTS

2 x 2 association studies among clinical features in individual NF1 patients

A major thrust is the characterization of associations between features of NF1 and mathematical modelling to quantify these relationships. We presented a preliminary analysis of two-way associations at the American Society of Human Genetics Meeting in Baltimore last October [1997].

We are now beginning to analyze more complex multi-way associations of clinical features in individual patients and their families. When we realized the complex relationship between age and the development of most symptoms, we restricted our analyses to children under 10 years in order to attempt to avoid confounding by age. As discussed below, age remained a confounding factor but the results of the studies were useful to direct our search for sub-types and were a necessary step in refining our technique. A sample table of some 2 x 2 analyses appears as appendix B and a detailed analysis of one such example is included in the next section, below:

Evaluation of the effect of age on associations among clinical features in individual NF1 patients

Many of the associations we observed in our comparisons of the occurrence of clinical features in individual NF1 patients probably result from confounding by age. The following example illustrates the complexities of dealing with the effect of age on the observed associations among features in NF1 patients. The age effect is illustrated on an association that was seen in individual patients, but the problem also affects our analysis of familial traits because the ages of relatives are often correlated.

We found a strong association between the presence of two common traits, Lisch nodules and cutaneous neurofibromatosis, among unrelated NF1 patients in the NNFFID (table 1).

Table 1 (all patients)		Lisch Nodules	
		Absent	Present
Cutaneous neurofibromas	Absent	679	532
	Present	161	530

Chi-square, 2-tailed probability, Yates' corr: p=.000001
Odds Ratio=4.2, (95% C.I. = 3.4-5.2)

The frequencies of both Lisch nodules and cutaneous neurofibromas increase with age. [Please see graphs of these two variables as a function of age, in appendix A, figures 1 and 2]. It is, therefore, likely that the association between these two features results from the fact that most younger NF1 patients do not have either feature and most older NF1 patients have both features. One approach to dealing with this problem is stratifying the analysis by age. Table 2 shows the same association for children under 10 years old.

Table 2
(<10 years)

		Lisch Nodules	
		Absent	Present
Cutaneous neurofibromas	Absent	499	197
	Present	48	41

Chi-square, 2-tailed probability, Yates' corr: p=.001
Odds Ratio=2.2, (95% C.I. = 1.3-3.5)

The association is still evident, but somewhat weaker. However, both of these features show substantial increases in frequency during the first 10 years of life. We still cannot eliminate age as the cause of this association.

If the analysis is limited to children under 5 years old (table 3), the association is no longer statistically significant, although the point estimate of the odds ratio is unchanged.

Table 3
(<5 years)

		Lisch Nodules	
		Absent	Present
Cutaneous neurofibromas	Absent	278	45
	Present	22	8

Chi-square, 2-tailed probability, Yates' corr: p=.1
Odds Ratio=2.2, (95% C.I. = 0.8-5.6)

The total number of patients included in this table is less than 20% of that in table 1, and the number of individuals in some of the cells has become quite small. As a consequence, we cannot be certain whether the association really no longer exists or we have just lost the statistical power to detect it.

A second approach to examining the effect of age is to consider only adults. Table 4 shows no association between these features in adults, but the cell sizes are even smaller and the statistical power even less.

Table 4
(30-50 years)

		Lisch Nodules	
		Absent	Present
Cutaneous neurofibromas	Absent	12	39
	Present	35	185

Chi-square, 2-tailed probability, Yates' corr: p=.3
Odds Ratio=1.6, (95% C.I. = 0.7-3.6)

Despite the problems of the technique, it does provide some useful information for our studies. These analyses can draw attention to interesting groups of patients. For example, very young patients with both Lisch nodules and cutaneous neurofibromas and adult patients with neither trait are exceptional. Such patients might represent an interesting clinical subgroup. Further evaluation of patients with "extreme" phenotypes, especially if they are familial, may lead to the recognition of unusually severe or mild forms of NF1.

For comparison, the same association was subjected to a log-linear analysis. For log-linear analysis, we chose the age categories used by Riccardi [1992, p28]: birth-6 mos,½ - 10 yrs, 10-12 yrs, 12-15 yrs, 16-30yrs,31-45 yrs and over 45yrs. Log-linear analysis is a two step process: The first step is to determine the best model, and the second step is to estimate the contributions to the overall variance that can be attributed to each parameter in the model. From this second series of calculations, the "true" odds ratio of each of the parameters in the model can be determined with the effects of confounding variables eliminated.

A hierarchical log-linear calculation with a saturated model and iterative proportional fit algorithm [SPSS ver 7.5.1, 1996] was used to determine the best log-linear model. Backwards elimination was used to remove higher order interaction terms that do not contribute to the model. The terms that remain are essential for the model fit. In the relationship between Lisch nodules, cutaneous neurofibromas, and age, there are no higher order interaction terms that contribute to the model: The only significant terms are the interactions between Lisch nodules and cutaneous neurofibromas; Lisch nodules and age; and cutaneous neurofibromas and age. The odds ratio can be directly determined from the parameter estimates: log$OR_{AB} = AB$ where is the probability of having Lisch nodules given the presence of cutaneous neurofibromas, and AB is model's parameter estimate for the pairwise association between Lisch nodules and cutaneous neurofibromas. The parameter estimate for the association between Lisch nodules and cutaneous neurofibromas is 0.7226, which gives an odds ratio of

2.06 (95% CI of 1.58 - 2.66). This approximation is very close to the odds ratio generated by manually stratifying the data (tables 2 through 4, above).

Both techniques (manually stratifying for age, and log-linear methods) treat age as a continuous variable. We have shown that either approach works well for categorical variables, but the different effects of age seen for many features seen in NF1 mean that it is inappropriate to treat age as a variable that has a uniform effect on the occurrence of different features at all ages. For this reason, we propose to use logistic regression (see further discussion of this in Problems and Proposed Solutions section, below).

Whether or not an association apparently exists between two traits, as in the sample association between Lisch nodules and cutaneous neurofibromas, table 1 (above), ascertainment bias must be considered. Ascertainment bias could either enhance or mask an association. Using the Lisch nodules/neurofibromas example, why do only 1902 patients (out of a database of over 3000 cases of confirmed NF1) have information recorded on Lisch nodules and cutaneous neurofibromas? The other 1000 patients are recorded as unknown. Is there a bias towards performing early slit lamp examinations in familial cases of NF1? Are more severely affected people more or less likely to receive slit lamp exams? Are some centres more or less likely to subject patients to slit lamp exams? In this case, examination of the responses shows no predilection for particular demographic variables, nor for contributing centres to respond in any particular way, and the likely explanation for the large number of unknown responses is that the slit lamp exam, necessary for diagnosis of Lisch nodules, is harder to perform on young children. This type of examination is carried out for each potential association.

Identification of clinical subgroups

We used our preliminary association studies, an extensive review of the literature, and the clinical experience of our investigators to identify a number of possible clinical subgroups among NF1 patients. These subgroups vary greatly in how firmly they are established as clinical entities. The existence of such a subgroup would imply patients who possess the feature (or associated set of features) differ in some biologically or pathogenetically important way from those who do not have the feature.

Some possible subgroups are shown in the following table. Our work has helped to define those subgroups marked with an asterisk*. We are most interested in identifying subgroups that show a consistent NF1 phenotype among affected members of a family, because these subgroups are most likely to result from particular mutations or classes of mutations of the *NF1* gene. Our plan is to chose 4 to 5 of these subgroups for our molecular case-control analysis after consultation with our collaborators. Phenotypes that are currently under strongest consideration for inclusion in the molecular case-control studies are marked in the following table with a double dagger‡.

Possible Subtypes of NF1 That Have Been Defined Clinically

- Large deletion phenotype* ‡
- Watson syndrome
- Familial café au lait spots
- NF-Noonan syndrome
- Familial paraspinal NF
- NF1 with familial malignant peripheral nerve sheath tumours ‡
- Gastrointestinal NF
- Late onset NF ‡

Possible NF1 Subgroups Suggested By Associations of Clinical Features

- Pulmonic stenosis* ‡
- Xanthogranulomas and juvenile chronic myelogenous leukemia*
- Optic glioma and other central nervous system gliomas* ‡
- Paraspinal tumours and superficial plexiform neurofibromas*
- Multiple subcutaneous and paraspinal neurofibromas* ‡
- Pseudarthrosis*
- Carcinoid of the duodenum and pheochromocytoma ‡
- Late diagnosed NF1, an initially mild phenotype* ‡

Possible NF1 Subgroups Defined on the Basis of Quantitative Abnormalities That May Occur As "All or None" Phenomena

- Macrocephaly* ‡
- Short stature*
- Early onset of puberty*

"Large deletion phenotype"

There have been a number of cases reported with a deletion of the entire *NF1* locus. Many of these patients have a more severe than usual phenotype with mental retardation or severe developmental delay, dysmorphic features, and early onset of discrete neurofibromas [Wu et al, 1995, 1996; Upadhyaya et al 1998]. However, there is controversy as to whether the presence of a deletion can be predicted from the clinical phenotype, or, alternatively, the observed association between this phenotype and deletions of the entire *NF1* locus is a result of bias of ascertainment [Tonsgard et al, 1997; Leppig et al, 1997; Wu et al, 1997; Valero et al, 1997; Upadhyaya et al, 1998; Rasmussen et al, 1998].

In view of this controversy, we decided to select patients that have the clinical phenotype associated with large *NF1* deletions as a test of our case-control methodology for establishing allele-phenotype correlations. We have identified sixteen

patients in the NNFFID with the phenotype described above and shall obtain blood specimens from as many of these patients as possible for *NF1* mutation analysis .

Watson syndrome

Pulmonic stenosis, multiple café-au-lait spots, and dull intelligence are the cardinal features of Watson syndrome [Watson 1967; Allanson et al. 1991]. Affected patients may also have other manifestations of NF1, including axillary freckling, Lisch nodules, multiple neurofibromas, short stature, macrocephaly, and characteristic "UBOs" on MRI scan of the brain [Watson 1967; Allanson et al. 1991; Leão and Robeiro da Silva 1995].

Watson syndrome is transmitted as an autosomal dominant trait with less phenotypic variability than is usually seen in NF1. Studies have demonstrated linkage of Watson syndrome to the *NF1* locus [Allanson et al. 1991], and an 80 kb deletion of the *NF1* gene has been reported in one patient with Watson syndrome [Upadhaya et al. 1992]. Thus, Watson syndrome appears to be an allelic variant of NF1.

Familial café au lait spots

Café-au-lait spots without other characteristic features of NF1 can be transmitted as an autosomal dominant trait [Whitehouse 1966; Riccardi 1982b; Charrow et al. 1993; Arnsmeier et al. 1994]. Linkage to *NF1* appears likely in one large kindred [Abeliovich et al. 1995]. The phenotype is consistent in affected members of this family: all have multiple café-au-lait spots and none has any other features of NF1. Other families with familial café au lait spots have been reported that do not exhibit linkage to the *NF1* locus.

NF- Noonan syndrome

Noonan syndrome is characterized by short stature, typical facies, broad or webbed neck, an unusual sternal deformity, cryptorchidism, and congenital heart disease [Allanson 1987; Sharland et al. 1992a; Noonan 1994]. Most patients with Noonan syndrome do not have NF-1 [Sharland et al. 1992a], and linkage to the *NF1* locus on chromosome 17 is unlikely in most families studied [Sharland et al. 1992b; Flintoff et al. 1993; Edman Ahlbom et al. 1995; Bahuau et al. 1996, 1998].

Features diagnostic of Noonan syndrome occur much more frequently than expected among NF-1 patients [Allanson et al. 1985; Mendez 1985; Opitz and Weaver 1985; Quattrin et al. 1987; Stern et al. 1992; Meschede et al. 1993; North 1993; Tassabehji et al. 1993; Colley et al. 1996; Carey 1998]. The concurrence of NF-1 and Noonan syndrome has been reported both in sporadic cases and in multiple members of some families [Quattrin et al. 1987; Stern et al. 1992; Tassabehji et al. 1993; Colley et al.

1996]. In the familial instances, the Noonan phenotype may not be observed in all relatives who have NF-1 [Stern et al. 1992; Colley et al. 1996].

Deletions of the entire *NF1* locus have been found in three unrelated families with NF-Noonan syndrome [Kayes et al. 1994; Colley et al. 1996]. An unusual in-frame 42-base tandem duplication of the *NF1* locus was observed in one other NF-Noonan syndrome family [Tassabehji et al. 1993]. Linkage of the NF-1 phenotype to the *NF1* locus was demonstrated in another family in which some members also have features of Noonan syndrome [Stern et al. 1992]. The most reasonable interpretation of these findings is that a mutation of the *NF1* locus can (but does not necessarily) produce a concurrent Noonan syndrome phenotype in some families.

Familial paraspinal NF1

Symptomatic paraspinal neurofibromas only occur in 2-4% of NF1 patients but are a common cause of neurological complications [Baser et al. 1998]. Asymptomatic lesions are much more common: paraspinal neurofibromas were found in 20-36% of adult NF1 patients on routine spinal imaging [Egelhoff et al, 1992; Tonsgard et al, 1998].

Familial cases of paraspinal neurofibromas have been reported by Pulst et al [1991], Poyhonen et al [1997a], and Ars et al [1998]. Ars et al. identified a frame-shift mutation that should theoretically result in a truncated protein product in her three-generation family. In the NNFFID, we have identified 45 patients with paraspinal tumours, including two families, each with two affected individuals.

Gastrointestinal NF

A few patients have been described who have multiple intestinal neurofibromas without other typical features of NF-1 [Heimann et al. 1988; Verhest et al. 1988; Shekitka and Sobin 1994]. The condition is transmitted as an autosomal dominant trait, although penetrance, at least for the intestinal manifestations, is incomplete. It is not clear whether or not mutations of *NF1* locus are involved in the pathogenesis of gastrointestinal NF.

Late onset NF

A few patients have been reported who have multiple discrete dermal neurofibromas but no café au lait spots or iris Lisch nodules [Riccardi 1982a, 1982b; Riccardi 1983]. These patients also lack most of the other typical features of NF-1. The neurofibromas may not appear until the third decade of life or later. The tumours may be cutaneous, subcutaneous, or both, and may be relatively localized or diffusely distributed.

The nature of this condition and its relationship (if any) to NF-1 is obscure. None of the reported cases has been familial, and, mutations of the *NF1* locus have not been demonstrated in any of these patients.

Pulmonic stenosis

Pulmonic stenosis, multiple café au lait spots, and dull intelligence are the cardinal features of Watson syndrome [Watson 1967; Allanson et al. 1991]. Pulmonic stenosis has been described in more than one affected member of three families with Watson syndrome [Watson 1967; Allanson et al. 1991; Leão et al. 1995]. The recognition that Watson syndrome results from mutations of the *NF1* gene raises the possibility that pulmonic stenosis may also occur with increased frequency among NF1 patients whose disease is more typical.

Observations in another phenotypic variant, the NF-Noonan syndrome, support this notion. Noonan syndrome is characterized by short stature, typical facies and congenital heart disease [Allanson 1987; Sharland et al. 1992; Noonan 1994]. Pulmonic stenosis occurs in about half of all patients with Noonan syndrome.

Although most patients with Noonan syndrome do not have NF1, and the disease is not linked to the *NF1* locus in most affected families, features diagnostic of Noonan syndrome occur much more often than expected among NF1 patients. Pulmonic stenosis has been observed in some patients with the NF-Noonan syndrome [Tassabehji et al. 1993; Colley et al. 1996].

Pulmonic stenosis is also the kind of congenital heart disease seen most often in NF1 patients without features of Watson syndrome or Noonan syndrome. Colley and associates [1996] found pulmonic stenosis in 9 (2.0%) of 453 NF1 patients. Four of the patients with pulmonic stenosis had neurofibromatosis-Noonan syndrome and two had Watson syndrome. Three NF1 patients had features of neither the Watson nor Noonan phenotype.

Our collaborator, Dr. D. Viskochil, reports an unpublished finding of a three base pair deletion in the *NF1* gene in four of six children in a Utah family. All four children have café au lait macule and features of Noonan syndrome. Two of the four children have pulmonic stenosis.

Pulmonic stenosis was observed in at least 15 of 2550 NF1 patients from the National Neurofibromatosis Foundation International Database [Friedman et al. 1997]. Individuals diagnosed as having Watson syndrome were excluded from this analysis. The frequency of pulmonic stenosis among these patients was 11 times greater than that expected in studies of the general population.

An interesting additional observation in this study is that 12 of the 15 NF1 patients with pulmonic stenosis were male [Friedman et al. 1997]. All four of the patients with NF1 and proven valvular pulmonic stenosis reported by Kaufman et al. [1972] were male, and most patients reported with Watson syndrome or neurofibromatosis-Noonan syndrome and pulmonic stenosis are also male [Allanson et al. 1991; Tassabehji et al. 1993; Leão et al. 1995].

We are performing more extensive studies on the patients with pulmonic stenosis and other cardiac malformations in NNFFID. We have requested, and have received some additional information on these patients from the NNFFID contributing centres. This information, which includes pedigrees with cardiac status in other affected and unaffected family members as well as data from specialized cardiac tests (ECG, echocardiograms, cardiac cathetarizations, angiograms and surgical reports) will help to better describe the phenotype of our pulmonic stenosis subgroup and to select cases for mutation analysis. We intend to request blood for mutation analysis on about 10 of our NF1- pulmonic stenosis patients, beginning in November, 1998.

Xanthogranulomas and juvenile chronic myelogenous leukemia

Watanabe et al, 1998; Tan et al, 1998; Zvulunov et al, 1995; and Morier et al, 1990, are amongst those that have proposed a link between Xanthogranulomas and juvenile chronic myelogenous leukemia. In the NNFFID there are numerous cases of xanthogranuloma (about 2.5% of children under the age of 10 have this condition, [Friedman et al, 1997c) but only one has of juvenile CML. If xanthogranulomas are a clinical marker for children at risk to develop CML by some common genetic mechanism, it is possible that the genotype of xanthogranuloma patients may have the same mutation type. These xanthogranuloma patients are therefore another possible sub-group of interest for mutation analysis.

Optic glioma and other central nervous system gliomas

Our own studies [Friedman et al, 1997b], and those of Kuenzle et al [1994], have suggested that some children with NF1 and an optic glioma are at increased risk for a second CNS tumour. In Kuenzle's longitudinal study, 11 of 21 (52%) children were diagnosed with a second tumor outside the optic pathway at a mean age of 4.0 years after the original diagnosis of optic glioma. In our own cross-sectional study, we were able to show that this association is not dependent on the effect of age or gender, nor is it associated with other features of NF1. Furthermore, no association is seen between optic glioma and non-CNS neoplasms. The association of optic glioma and other intracranial neoplasms in patients with NF1 suggests that there are fundamental pathophysiological differences between patients with and without optic glioma. This

potential seriousness of these tumours makes this group of patients an important subgroup to subject to molecular analysis.

Paraspinal tumours and superficial plexiform neurofibromas

We have begun analyzing a set of patients who have extensive paraspinal neurofibromas, a potentially very serious complication of NF1. Paraspinal tumours probably in occur in at least 1 in 5 adult patients with NF1 [Tonsgard et al, 1998; Egelhoff et al, 1991], but the majority of these patients are asymptomatic.

We analyzed data from the NNFFID and from Dr. Evans' database which has a higher percentage of older patients. We age- and sex-matched a group of 45 NNFFID patients with paraspinal neurofibromas with those without paraspinal neurofibromas. In all age groups of the NNFFID data, 3.3% of patients with superficial plexiform neurofibromas had paraspinal tumours versus 1.3% of patients without superficial plexiform tumours (OR = 2.6, 95% CI = 1.4 - 4.9). In patients under 16 years of age, this association appeared to be even stronger (OR = 5.4, 95% CI = 2.0 - 15.2).

In the Evans database, the strength of the association between superficial plexiform neurofibromas and paraspinal neurofibromas was similar, although statistical significance was not seen in this smaller set: OR = 3.4 (95% CI = 0.9 - 13.1). There were no associations seen between paraspinal tumours and other features recorded in the database, nor did the covariates of gender or race interact with the association [Baser et al, submitted for publication, 1998]. We intend to continue these studies in the Riccardi database and to evaluate the association in all three data sets using logistic regressive techniques to adequately account for the effect of age.

The approach we are taking illustrates the value of using three different large NF1 databases in parallel: one database is used to generate pathogenic hypotheses, and the other two are used to test these hypotheses. Morever, each database has its own particular strengths, and the use of all three together enables us to avoid some of the statistical limitations inherent in this kind of research.

Multiple deep neurofibromas and malignant peripheral nerve sheath tumours

Most malignant peripheral nerve sheath tumours appear to arise from preexisting deep nodular or plexiform neurofibromas [Wander & Das Gupta, 1977; Riccardi & Powell, 1989; Riccardi 1992]. It is, therefore, reasonable to presume that the risk of developing a MPNST is greater in NF1 patients who have many deep neurofibromas than in NF1

patients who do not. Although this presumption is consistent with the clinical experience of physicians who have seen many NF1 patients [VM Riccardi, personal communication; GR Evans, personal communication], it has not been tested formally.

The possible associations between superficial plexiform neurofibromas and multiple paraspinal tumours (discussed above) and between multiple deep neurofibromas and MPNSTs suggest that it may be possible to identify a subgroup of NF1 patients who are at higher risk to develop some of the most devastating complications of NF1 on the basis of more benign, preexisting lesions. It is uncertain whether such patients differ from others with NF1, but reports of familial occurrence of paraspinal neurofibromas [Pulst et al 1991; Poyhonen et al 1997a and 1997b; and Ars et al 1998; see also "Familial paraspinal NF" above] are consistent with this interpretation.

Pseudarthrosis

We have collaborated with Dr. John Carey's group, University of Utah, on a description of the natural history of pseudarthrosis [Stevenson et al, in press]. 85 of 1479 unrelated individuals with NF1 and tibial pseudarthrosis were compared 2:1 to a control group of NF1 patients without NF1. Of the pseudarthrosis group, 54 were male and 31 were female (chi-square with Yates' correction for continuity = 3.7; p=.05). In the group with tibial bowing alone, there were 18 males and 15 females whereas in the group with fractures, 36 were male and 16 were female (chi-square with Yates' correction for continuity = 4.5; p=.03; OR= 2.2; 95% CI = 1.1-4.9). There were no differences in the frequencies of any other features of NF1, nor was there any apparent parent of origin affect. It is interesting to note that both pseudarthrosis and pulmonic stenosis appear to have a male predominance in NF1 whereas neither has a sex bias in the general population.

Carcinoid of the duodenum, pheochromocytoma and NF1

Pheochromocytomas are rare tumours, but they occur more often in NF1 patients than in others. The incidence in the general population is estimated to be about 0.4 per 100,000 per year [Wu et al. 1997]. The frequency among NF1 patients in most series is 0.1-1.5%, with higher frequencies in older groups of patients [Mulvihill 1994; Zöller et al. 1997].

Even benign pheochromocytomas in NF1 patients usually show loss of heterozygosity for DNA markers in the region of the *NF1* gene [Ponder et al., 1990]. Loss of heterozygosity in this genetic region is uncommon in pheochromocytomas from patients who do not have NF1. This observation suggests that the pathogenesis of

pheochromocytoma in NF1 patients may differ from that in patients who do not have neurofibromatosis.

Carcinoid tumours are neoplasms that contain a variety of peptide hormones. Carcinoid tumours occur in the general population with an incidence of about 1.5 cases per 100,000 per year [Vinik and Perry 1997] but are more common among NF1 patients [Burke et al. 1990; Scully et al. 1989; Mulvihill 1994]. Reported carcinoid tumours in NF1 patients usually arise in or near the ampulla of Vater [Griffiths et al. 1987; Ferner 1994], but tumours in this location may be more likely to produce jaundice and come to medical attention.

Almost all carcinoid tumours that have been studied from NF1 patients contain somatostatin [Griffiths et al. 1987; Burke et al. 1990] and can, therefore, be classified as somatostatinomas. The carcinoids that arise in NF1 patients have a distinctive histopathological appearance [Griffiths et al. 1987; Burke et al. 1990]. NF1 patients may have multiple primary carcinoid tumours [Hough et al. 1983; Dawson et al. 1984; Burke et al. 1990].

NF1 patients who have a pheochromocytoma are especially likely to have a carcinoid tumour as well, and vice versa [Wheeler et al. 1986; Griffiths et al. 1987]. In one series of 27 NF1 patients with duodenal carcinoid tumours, 6 (22%) also had pheochromocytoma [Griffiths et al. 1987]. This association may reflect a propensity of people with certain *NF1* mutations to develop both neoplasms.

Late diagnosed NF1, an initially mild phenotype

We have identified an interesting possible subtype of NF1 amongst the sporadic cases in NNFFID [DeBella et al, 1998]. This group consists of patients who met only one of the NIH diagnostic criteria for NF1 when initially evaluated at an average age of 1.6 years. 41 of these patients eventually developed other signs of neurofibromatosis and were diagnosed with NF1. Seven of the patients could not be diagnosed until after age 6, and one was not diagnosed until age 19 (Please see appendix A, figure 7). This late diagnosis of NF1 contradicts conventional clinical wisdom that states the diagnosis of NF1 can almost always be made with a thorough clinical and ophthalmological exam by the age of 6 [Obringer et al., 1989; Korf, 1992]. We intend to perform a more thorough analysis of this phenotype, including searching for similar patients in our other two databases. We are particularly interested in determining whether this late diagnosis is a familial trait in NF1.

Macrocephaly

Macrocephaly is well-recognized feature of NF1, but the prevalence, natural history, and prevalence of macrocephaly among NF1 patients has not been well characterized. Riccardi [1992, 1993] suggests that macrocephaly is an "all or none" phenomenon in NF1, i.e., a discontinuous trait that is either present or absent in a particular patient. If this is correct, NF1 patients with macrocephaly may represent a pathogenetically different group from NF1 patients without macrocephaly.

We studied head circumference measurements of 2958 children with NF1 from the NNFFID and compared the findings to normal population standard for children of the same sex and age by plotting centiles by age and by using z-scores based on the normal standards [Szudek et al., 1998]. Distributions of z-scores were quantified by plotting their cumulative frequency distributions on a normal probability scale to look for bimodality, according to the method of Goldstein et al [1973] (appendix C, figure 1).

The plots of head circumference and head circumference/height ratio by age among children with NF1 (appendix C, figures 2 and 3) show that, on average, NF1 patients have bigger heads than normal populations throughout childhood. Our data do not support macrocephaly as an "all or none" phenomenon in NF1 because the distribution as a whole appears to be shifted upward -- there is no evidence of a group with normal head circumferences for age and another group with macrocephaly (appendix C, figure 1). However, the standardized distribution of head circumference/height measurements in NF1 patients differs significantly from a Gaussian distribution, and there is some evidence for a second mode (i.e., a small subgroup) among patients with the very highest standardized values (appendix C, figure 4).

Short stature

Short stature is also commonly observed among NF1 patients. Riccardi [1992, 1993] has also suggested that short stature is an "all or none" phenomenon in NF1. We used methods identical to those described above for macrocephaly to study height measurements of 2958 children with NF1 from the NNFFID [Szudek et al., 1998]. We excluded patients with premature or delayed puberty, pseudarthrosis, scoliosis, vertebral dysplasia, or spinal compression from analyses to avoid confounding with pathological lesions that are known to affect growth.

Centile plots of height showed that NF1 patients are shorter on the average than unaffected children of the same sex and age, as expected (appendix C, figure 5). The observed distributions for height are Gaussian and unimodal (appendix C, figure 6);

there is no evidence of two different subgroups. The mild linear growth deficiency characteristic of NF1 appears to affect all patients to some degree.

Early onset of puberty

We are also studying a group of patients with unexplained precocious puberty. Precocious puberty is known to be associated with optic chiasm gliomas in NF1 patients. Out of 2550 patients, 61 have precocious puberty. Among these, 17 have known optic glioma and 11 have hypothalamic-pituitary pathology. At least 8 of the remaining patients have had MRI scans that show no evidence of brain lesions. The frequency of precocious puberty without brain lesion in NNFFID is therefore 0.3%, as opposed to a population incidence of about 0.06%. ($p<.05$). Our numbers are small, however and we are requesting additional information from our contributors to characterize this interesting subset of patients better as well as to determine the status of the 25 patients with precocious puberty but unknown scan results. The observation of a potential association between precocious puberty and NF1 in the absence of optic glioma has been previously reported [Zacharin et al, 1997; Cnossen et al 1997].

DISCUSSION - PROBLEMS AND POTENTIAL SOLUTIONS

In the sample association described in Methods (above) we observed an association between the presence of cutaneous neurofibromas and Lisch nodules using either manual stratification for age, or log-linear modelling. However, the frequency of each of these features increases rapidly within certain age groups (figures x and y), so the observed association may be a result of confounding -- an adult with NF1 is more likely to have both Lisch nodules and neurofibromas simply because both features are present in most adults with NF1; a young child is likely to have neither simply because neither feature is common in young children. The usual methods of dealing with this problem are to stratify the analysis by age categories (e.g. 0-5 yrs, 6-10 years...) or to adjust for the variable of concern (in this case, age) by treating it as a covariate in the statistical model. These were the approaches we planned to take using log-linear methods [Beaty, 1991; other ref] to account for patients's ages.

However, after analysing our preliminary results and discussing them with Dr. Muin Khoury, our epidemiology consultant, it became evident that we could not reliably eliminate the confounding by age use these methods with log-linear modelling. We cannot reliably use a age-stratified analysis because the rate of change with age varies so much among clinical features, particularly in childhood, that the age categories would have to be very small to approximate the homogenous group necessary for valid analysis. Even with a database of over 3500 cases, this results in cell sizes too small for statistical reliability.

The differing rate of change with age among features also vitiates our ability to adjust the log-linear analysis by treating age as a covariate. This adjustment assumes a uniform effect of age on the occurrence of both features, an assumption that is clearly invalid for most combinations of features in NF1.

In order to illustrate the problem completely, we graphed all variables by age. The frequencies of many traits increase with age throughout childhood and level off in adulthood. However, the rates of increase, the maximum frequency, and the age at which the maximum is reached vary greatly among features. Other traits follow completely different patterns: e.g., café au lait macule reach their maximum frequency by 1 year of age, U.B.O.s and xanthogranulomas initially increase, then decrease in frequency during childhood and adolescence, and malignant neoplasms to not reach their maximum frequency until late adulthood. A sample of these graphs appears in the appendix A (figures 1-6)

Another possible approach to this problem is to limit our analysis to adult patients, in whom the effects of age on most trait frequencies is less marked. This method has several disadvantages: Firstly, any subgroups that are associated with significant

mortality would be less represented in the adult population as a result of premature death. In one prospective pediatric follow-up study, the childhood mortality was over 3% [Cnossen et al, 1998]. Clinically, these phenotypes are the most important sub-groups to identify because they represent more severe disease. Secondly, we are anxious to identify phenotypic subgroups early in life that are highly predictive of the occurrence (or non-occurrence) of particular complications later in life. Recognizing such subgroups would be useful in patient management and genetic counselling. A third difficulty is one of sample size: The database has fewer adults than it does paediatric patients (62% of the patients are under the age of 16 years), so any analytical technique will be limited by problems of statistical power, particularly for some of the less common, and often more serious, features of NF1.

A more flexible approach for analysis of combinations of features that vary with age is to use logistic regressive modelling [Draper & Smith, 1966; Hosmer & Lemeshow, 1989]. This method offers several advantages. It is appropriate for binary outcome variables, which include most of the important complications of NF1. Logistic regression can be used to estimate odds ratios that are adjusted for multiple risk factors, including both continuous, binary, and categorical variables. Interactions among risk factors can also be explicitly included in the model, and their importance assessed. In addition, well-established methods exist for extending logistic regressive analysis to include genetic effects [Liang Y Beaty, 1991; Piegorsch et al., 1994; Karunaratne & Elston, 1998], a major concern of this project.

Logistic regressive methods were originally designed for situations in which the logarithm of the odds of disease occurrence is a linear function of the risk factor, a situation that clearly does not apply to most serious complications of NF1. However, the methods can accommodate nonlinearity of the response by using transformations of the relationship between the risk factor and outcome variable into a form that fits the data [Draper & Smith, 1966; Hosmer & Lemeshow, 1989]. Once an appropriate logistic model has been established, the impact of individual risk factors on the occurrence of the outcome variable can be estimated by means of the adjusted odds ratio [Selvin, 1991].

CONCLUSIONS

The clinical manifestations of NF1 are extremely variable. The purpose of this project is to characterise the sources of phenotypic variability in NF1 by using a combination of clinical, statistical, epidemiological, and molecular genetic methods. During the first year of this project, our goals were to identify subgroups of NF1 patients in which at least some of the phenotypic variability appears to result from genetic factors and to set up the molecular techniques necessary for identification of up to 90% of *NF1* gene mutations.

We have initiated our collaborations with Doctors Riccardi, Viskochil, and Khoury. We have also undertaken a collaboration with Dr. Gareth Evans who has provided us access to an additional database of over 500 NF1 patients from Dr. Gareth Evans. This database, which is population-based and contains more complete families and more adult cases than either the NNFFID or the NF Institute Database, is a valuable complement to our existing data resources.

We began our analysis by producing two-by-two tables for the approximately 100 features in the NNFFID. We then examined potential associations for covariates using methods appropriate for each covariate. When we examined the effect of age on various associations, we realised that age does not have a uniform effect on each variable and that this may substantially complicate the analysis. In order to explore this problem more fully, we graphed the prevalence of all clinical features of NF1 among patients in the NNFFID by age. We are using several approaches to deal with the very important but non-linear confounding effect of age on our analysis, depending on how age affects a particular set of variables. These methods include stratified analysis, log-linear modelling, and logistic regression.

We have used these and other methods to develop a list of possible clinical subsets of NF1 patients to determine if they comprise appropriate groups in which to pursue genotype-phenotype correlations. These patients have unusual features of NF1 that might be indicative of distinctive pathogenic mechanisms. Our working hypothesis is that variations in pathogenesis may reflect variations in the specific mutations present in the NF1 gene. We are currently analysing several of these subsets by a combination of clinical, genetic, and statistical approaches to determine which are most likely to

represent allelic variants of NF1. Our current provisional list of most likely candidate phenotypes includes the following:

- Large deletion phenotype
- NF1 with familial malignant peripheral nerve sheath tumours
- Late onset NF
- Pulmonic stenosis
- Optic glioma and other central nervous system gliomas
- Multiple subcutaneous and paraspinal neurofibromas
- Carcinoid of the duodenum and pheochromocytoma
- Late diagnosed NF1, an initially mild phenotype
- Macrocephaly

Our collaborator, Dr. David Viskochil has implemented most of the molecular techniques required for the strategic screening process we shall use for identification of constitutional mutations of *NF1*.

During the second year of the project, we shall begin testing selected phenotypic subgroups of NF1 patients using our case-control design to determine if allelic differences in the *NF1* gene are likely to account for the particular clinical features used to identify the subgroup. We shall begin with the so-called "large deletion phenotype" and continue with other phenotypes selected by a combination of clinical, genetic, and statistical methods in consultation with Drs. Khoury, Riccardi, and Viskochil.

<u>Technical Objective #6:</u> Set up techniques for strategic screening process for identification of constitutional mutations of *NF1* .

This technical objective is encompassed in the schematic diagram shown in figure 1 which is a revision of the algorithm provided as figure 1 in the original proposal. There have been two modifications to the original proposal. RNA extracted from whole blood yielded variable results in the RT-PCR (reverse transcription followed by long polymerase chain reaction) analysis depicted as step 6 in the schematic. Therefore, we have obtained approval to transform lymphocytes through the Clinical Research Center at the University of Utah. It is a long-standing facility funded through an NCRR grant, #M01-RR00064 (Principal Investigator – James Kushner). Second, we have not been pressed to perform interphase FISH (fluorescence *in* situ hybridization) as shown in step 3 of the algorithm. Access to tissue culture and our ability to perform metaphase spreads in the Core FISH Facility has made steps 4 and 8 a straightforward routine. Therefore, we now perform metaphase FISH on all samples that demonstrate lack of heterozygosity by genotype analysis.

1. RNA extraction
2. DNA extraction
3. Cell preparation
4. Lymphocyte culture
5. Genotype analysis
6. cDNA synthesis
7. Interphase FISH
8. Metaphase FISH
9. Long PCR of cDNA
10. Restriction enzyme digestion analysis
11. Exon segment PCR
12. IVTT
13. PAGE analysis
14. Size-shift analysis

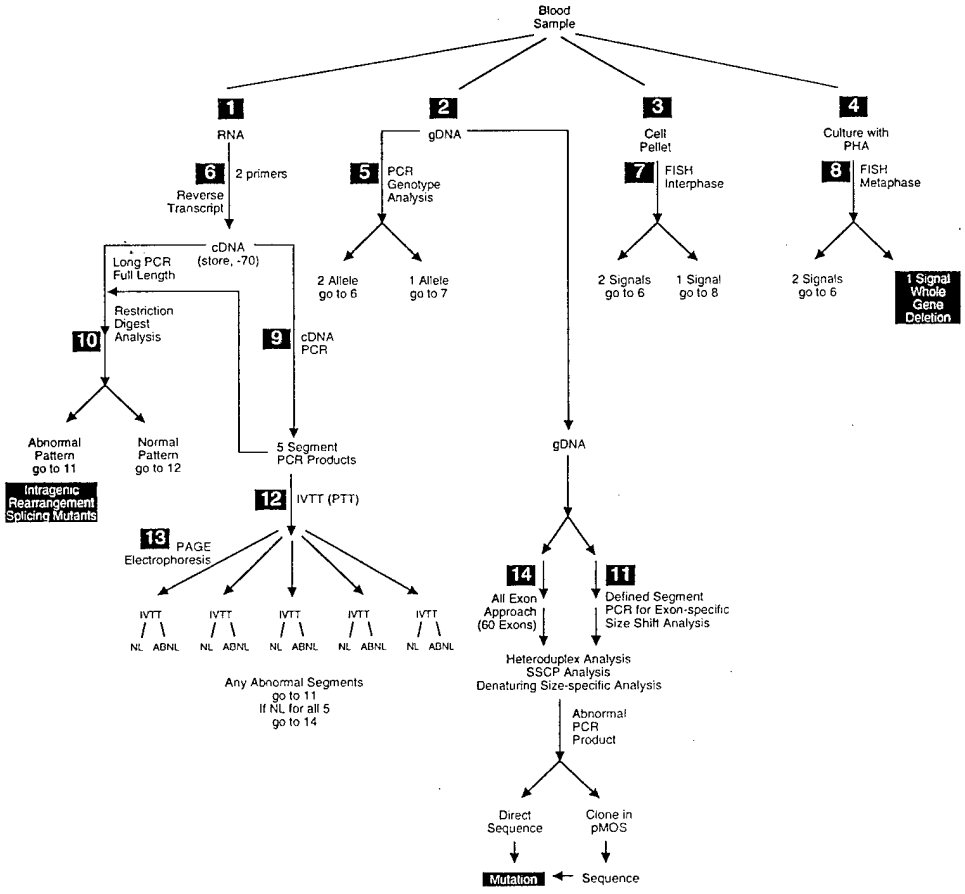


Figure 1. Algorithm depicting the strategic plan to identify *NF1* mutations.

The methodology to triage blood samples for *NF1* mutation analysis is in place. Blood samples are collected in ACD-vacutainer tubes and mailed by overnight express to Dr. Viskochil's laboratory. Upon arrival the tubes and clinical information are "logged in" and provided a lab number as a unique identifier before dissemination. One tube is processed for DNA extraction, one is processed in the Clinical Research Center (CRC) at the University of Utah for Epstein-Barr viral (EBV) transformation, and one is processed for routine short-term lymphocyte culture for FISH (fluorescence *in situ* hybridization) analysis. The EBV-transformation takes approximately 4-6 weeks and approximately 80% of samples actually are immortalized on the first attempt. The cell lines then serve as a renewable resource for RNA-based *NF1* cDNA mutation analysis. The CRC stores the "transformed" cells, and upon request provides the frozen cells to the Viskochil laboratory. Using routine techniques, the cells are thawed and expanded in tissue culture. Two T-75 flasks are used for RNA extraction. The quantity and quality of extraction is determined by spectroscopy. The applicability of DNA extraction from transported blood samples has been simply demonstrated by spectroscopic analysis. The integrity of the sample is also gauged by its robustness as DNA template in PCR.

The initial step in mutation analysis is to screen for a constitutional deletion. We have implemented mutagenically-separated PCR (MS-PCR) analysis at three single nucleotide polymorphism (SNP) loci in the *NF1* exon sequence. Figure 2 depicts MS-PCR products for SNP 702 (exon 5), SNP 2034 (exon 13), and SNP 10467 (exon 49). Readout from this assay is available within 72 hours of receipt of the sample. In a sample cohort, 18 of 34 Japanese NF1 patients were heterozygous at one or more loci. Therefore, approximately half of all samples can be shown to not harbor a large gene deletion within three days of sample proceessing. The presence of only one allele at all three sites, defined as non-heterozygosity, directs the screening methodology to FISH analysis of the blood sample which originally set up for culture upon receipt of the blood sample.

Figure 2. Example of MS-PCR to determine heterozygosity at the *NF1* locus; a=a/a homozygous or a/- hemizygous; g=g/g homozygous or g/- hemizygous; h=heterozygous.

Cultured lymphocytes are routinely processed for metaphase spreads by standard methodology. FISH is performed using two probes simultaneously, P1-9 (*NF1* exons 2-11) and P1-U (mapped to 17q25). Different fluors are incorporated in the probe to allow dual fluorescence. An image showing loss of one *NF1* allele is shown in figure 3. This patient has multiple neurofibromas, malignant peripheral nerve sheath tumor, and mental retardation. He has also experienced other rare complications of NF1, however he has not yet been characterized as having a distinct phenotype through cluster analysis.

Figure 3. FISH analysis of 94-04-21. There is a loss of the P1-9 signal on one chromosome 17.

In the event two alleles are identified in MS-PCR, the mutation analysis algorithm shifts to either cDNA or genomic DNA analysis. Genomic DNA analysis techniques have been adopted for muliplex size-shift analysis. In this technique, all 60 exons are amplified and electrophoresed to separate exon-based PCR products that harbor a mutation. An example of this analysis is shown in figure 4. We are now attempting to increase the number of PCR products in a multiplex PCR synthesis of up to 14 exon-based PCR products in one reaction. As depicted in figure 4, the radioactive products (end-labelled by incorporation of ^{33}P-labelled primer into the PCR product) are shown as a copy of an autoradiograph. It demonstrates a shift in the PCR product spanning exon 45. We have isolated the PCR product and show by sequence analysis (see figure 5a) that this NF1 patient has a mutation in intron 45 that leads to alternative splicing of *NF1* mRNA resulting in a frame-shift that predicts premature truncation of translation (see figure 5b). Using the size-shift analysis another *NF1* mutation has been identified in a family who has multiple members with café-au-lait spots and pulmonary valve stenosis. There is a band

shift in exon 17 which has been sequenced to demonstrate a three-base deletion and dropout of an encoded methionine in the resultant peptide (see figure 6).

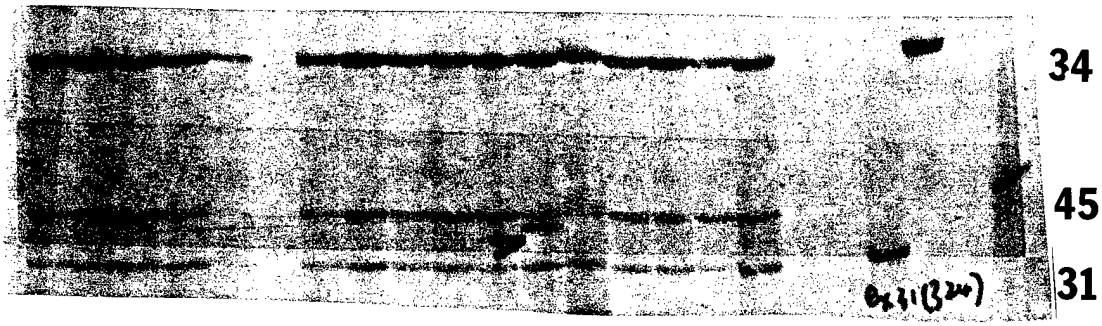


Figure 4. Autoradiograph depicting size-shift of exon 45 PCR product.

We are in process of applying techniques to capture multiple *NF1* exon PCR products amplified from genomic DNA onto a nylon membrane. Subsequent hybridization to full-length *NF1* cDNA probe would enable us to screen multiple PCR products as part of one gel electrophoresis run. This applicaton, if effective, would enhance band separation and enable us to convert from radioactive to non-radioactive methodology. This technique would also enhance cDNA screening by RT-PCR restriction digest methodology as covered in the grant proposal.

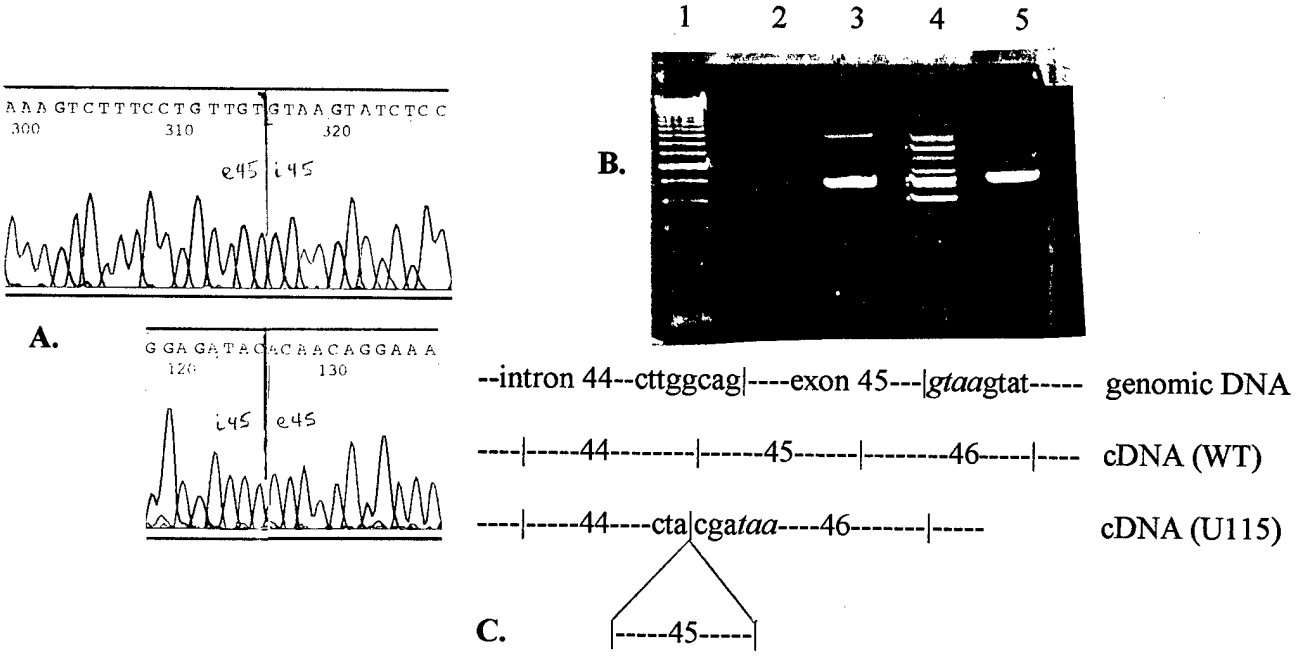
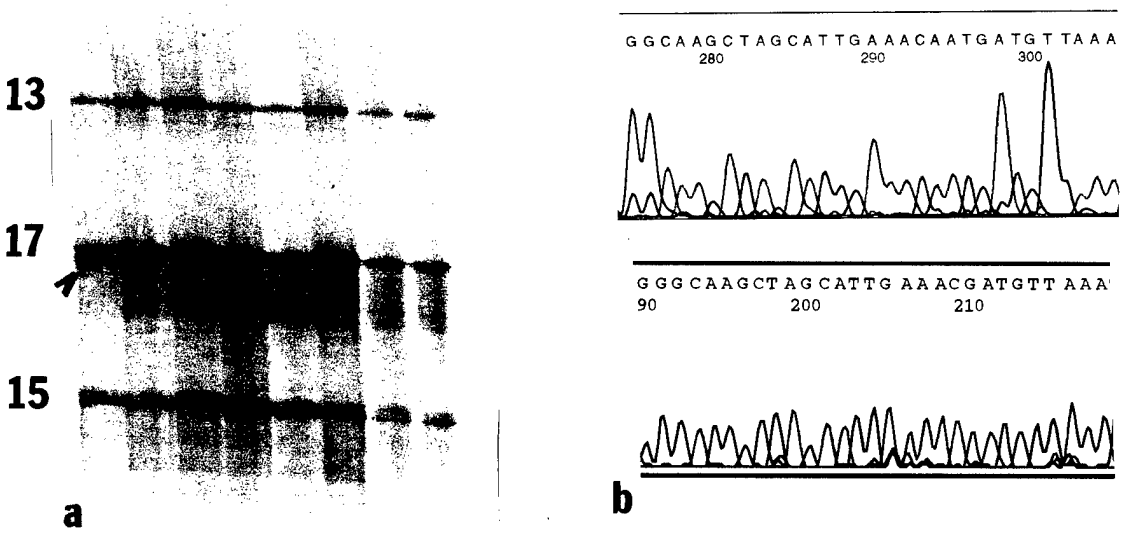


Figure 5. Mutation characterization of exon 45 PCR size-shift. A) DNA sequence of normal genomic sequence and the complement of the same region showing a 4-bp deletion in intron 45. B) RT-PCR of RNA showing abnormal mRNA splice variants (lane 1-marker, lane 2-water blank, lane 3-control RNA template for RT-PCR, lane 4-patient U115 RNA for RT-PCR, lane 5-PCR using cloned *NF1* cDNA as template). C) Demonstration of *NF1* mutation showing 4-bp deletion in intron 45, the splicing out of exon 45, and premature stop codon in cDNA.



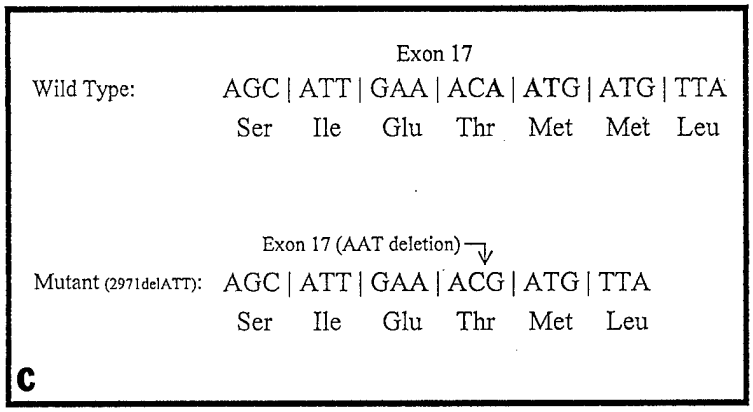


Figure 6. Characterization of size-shift variant from exon 17. a) autoradiograph of size-shift PCR product. b) sequence analysis of PCR product. c) resultant effect on peptide.

The application of mRNA-based techniques for *NF1* mutation analysis have been addressed even though a cohort sample has not been screened. Various techniques for RNA extraction and cDNA synthesis have been evaluated. Single primers specific for the 3'-end of the *NF1* gene have <u>not</u> been successful in providing RT-PCR (reverse transcription – polymerase chain reaction) products which are required for subsequent analysis. We have been forced to use random hexamer priming as our method to generate cDNA which can then serve as template for PCR using primers spaced approximately 2 kb apart in long PCR protocols. Figure 7 shows the RT-PCR products for all 5 segments of *NF1* cDNA.

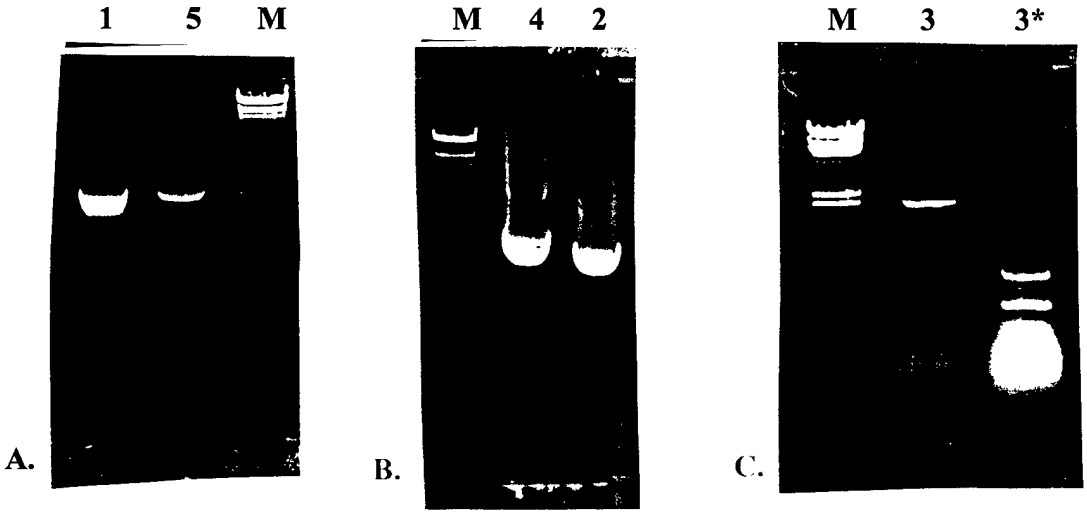


Figure 7. RT-PCR of five segments of the *NF1* cDNA. A) Primers 1F/1R (segment 1) and 5F/5R (segment 5). B) Primers 2F/2R (segment 2) and 4F/4R (segment 4). C) Comparison of RT-PCR when cDNA synthesis is primed with random hexamers versus an *NF1*-specific primer, 3R.

The RT-PCR segments can be evaluated for exon dropout which arise from splicing error mutations. We also propose to use these products to screen for cDNA fragment size shifts as depicted in step 10 of the algorithm. Table 1 is a list of selected restriction enzyme digest patterns that would result in fragment sizes easily identified in a size-shift assay. As a proof-of-principle, segment 3 was synthesized as a 2-kb RT-PCR product from blood RNA and digested with restriction enzymes to generate smaller DNA bands. These smaller DNA segments could then be screened by electrophresis migration to identify size-shifted bands similar to the exon-based PCR size-shift analysis from genomic DNA template. We have had difficulty in consistently amplifying segment 1. Nevertheless, as demonstrated here, the techniques of generating long-segment PCR products from *NF1* RNA are functional, even though they have not been applied in a systematic approach to defining a mutation in an NF1 patient cohort.

RT-PCR segment	restriction enzymes	fragment sizes of restriction digests (bases)
1 (-1 to 1868)	*Bsr*I, *Mam*I, *Sfu*I	116, 161,199, 276, 263, 854
2 (1486-3583)	*Ava*II, *Msp*I	25, 139, 148 (2), 183, 189, 231, 274, 378, 445
3 (3217-5255)	*Eco*RI, *Hae*III	149, 159, 200, 225, 234, 257, 320*, 361, 477
4 (4998-6988)	*Cac*81, *Pst*I, *Tfi*I	79, 111, 185, 326, 348, 365, 399, 414
5 (6574-8304)	*Ava*I, *Rsa*I	156, 190, 242, 279, 309, 355, 403

Table 1.

The application of the protein truncation test (PTT) by *in vitro* transcription and translation (IVTT) using cDNA template has not been fully implemented. The 5 primer sets harboring transcription and translation start signals have been synthesized and shown to amplify cDNA synthesized by Reverse Transcriptase. It will be incorporated in the screening process when patient cohort samples are collected.

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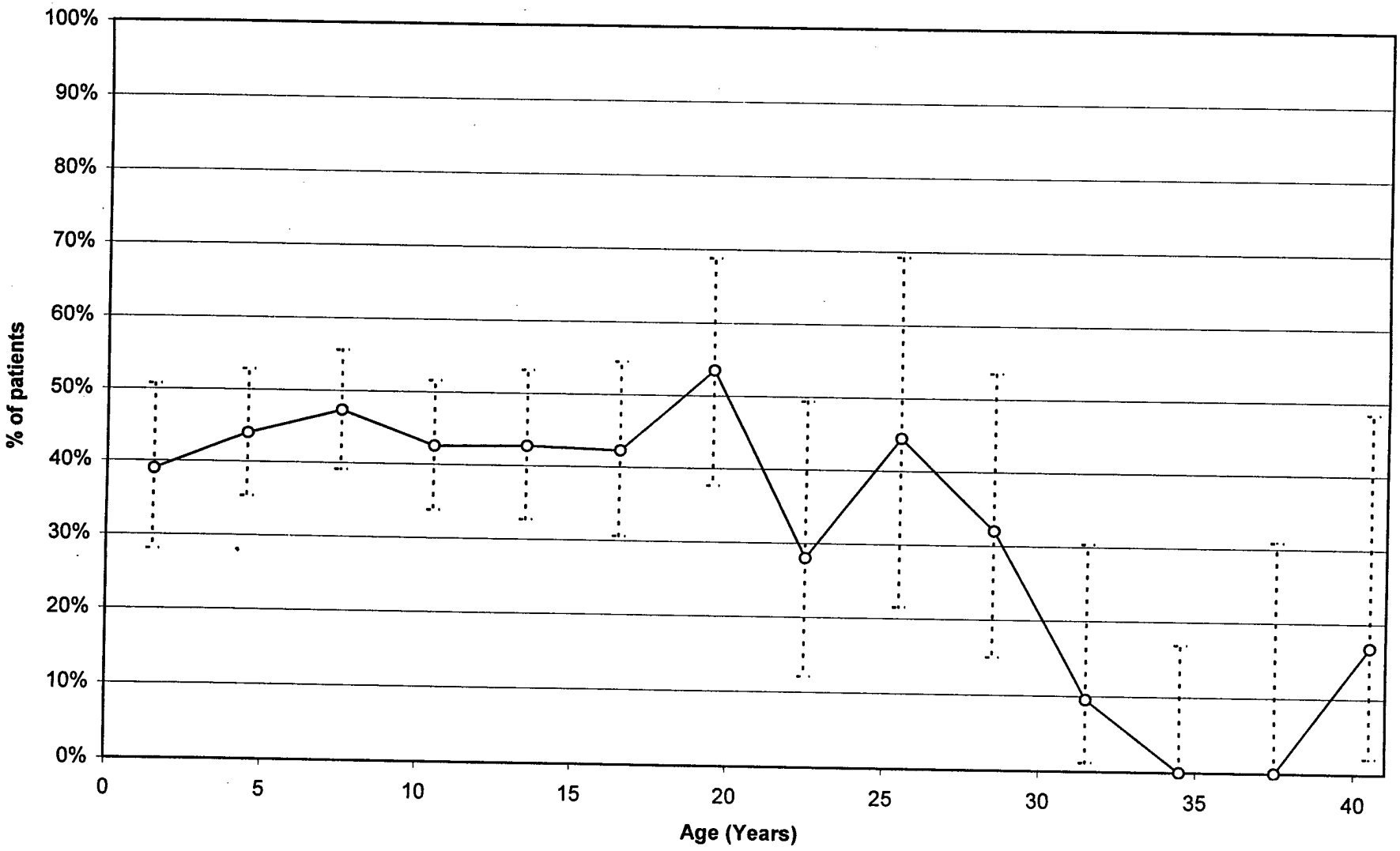
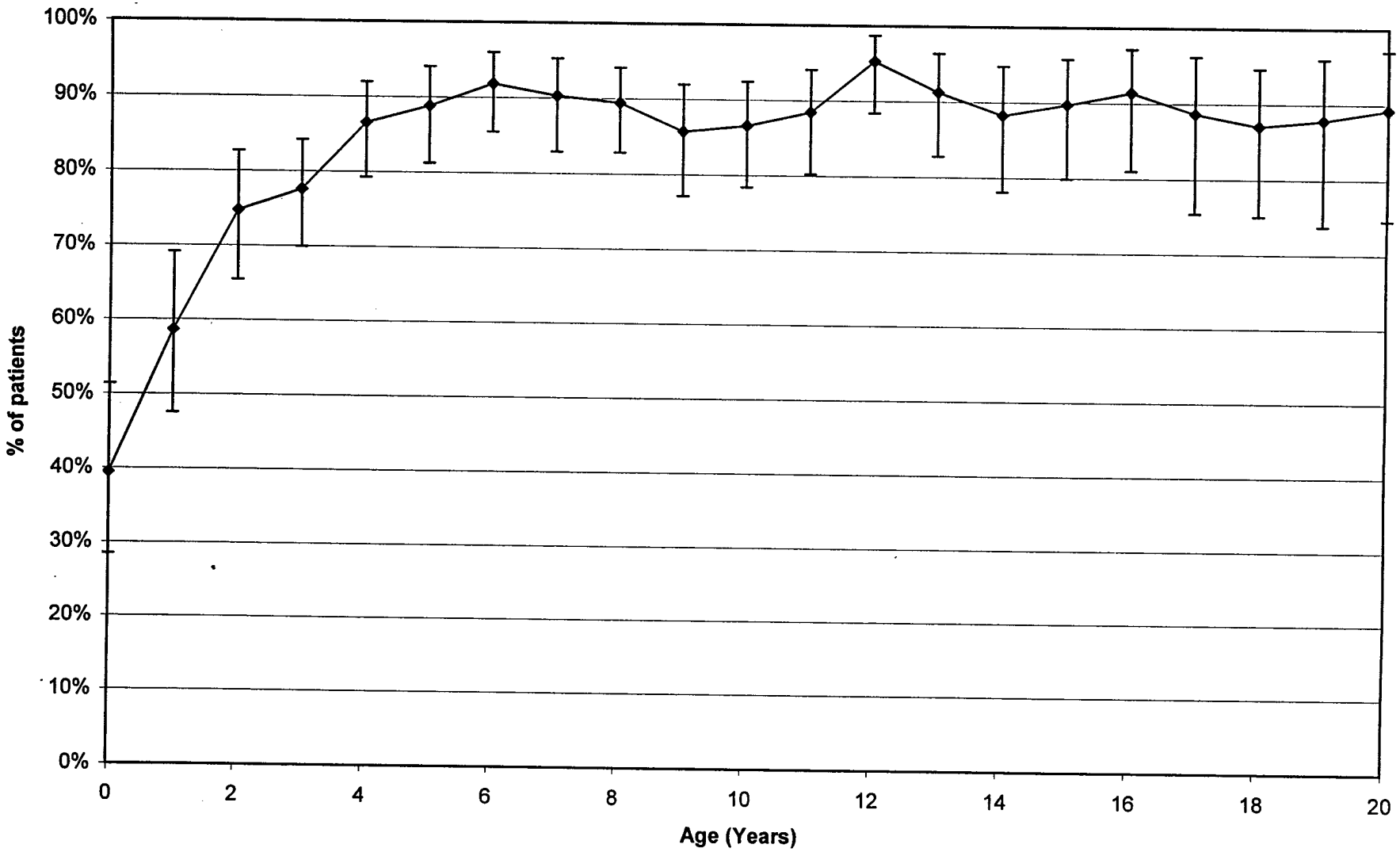
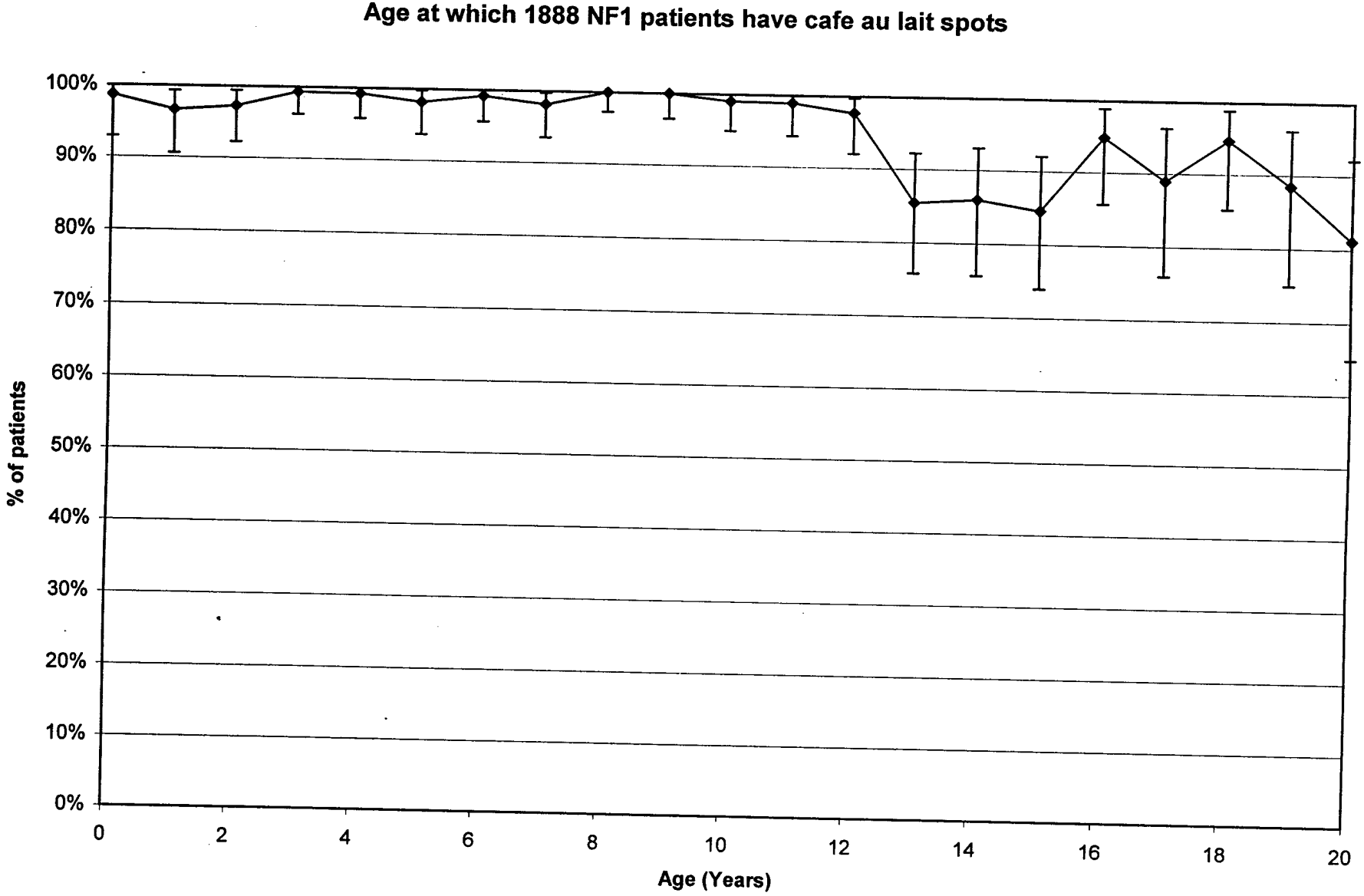
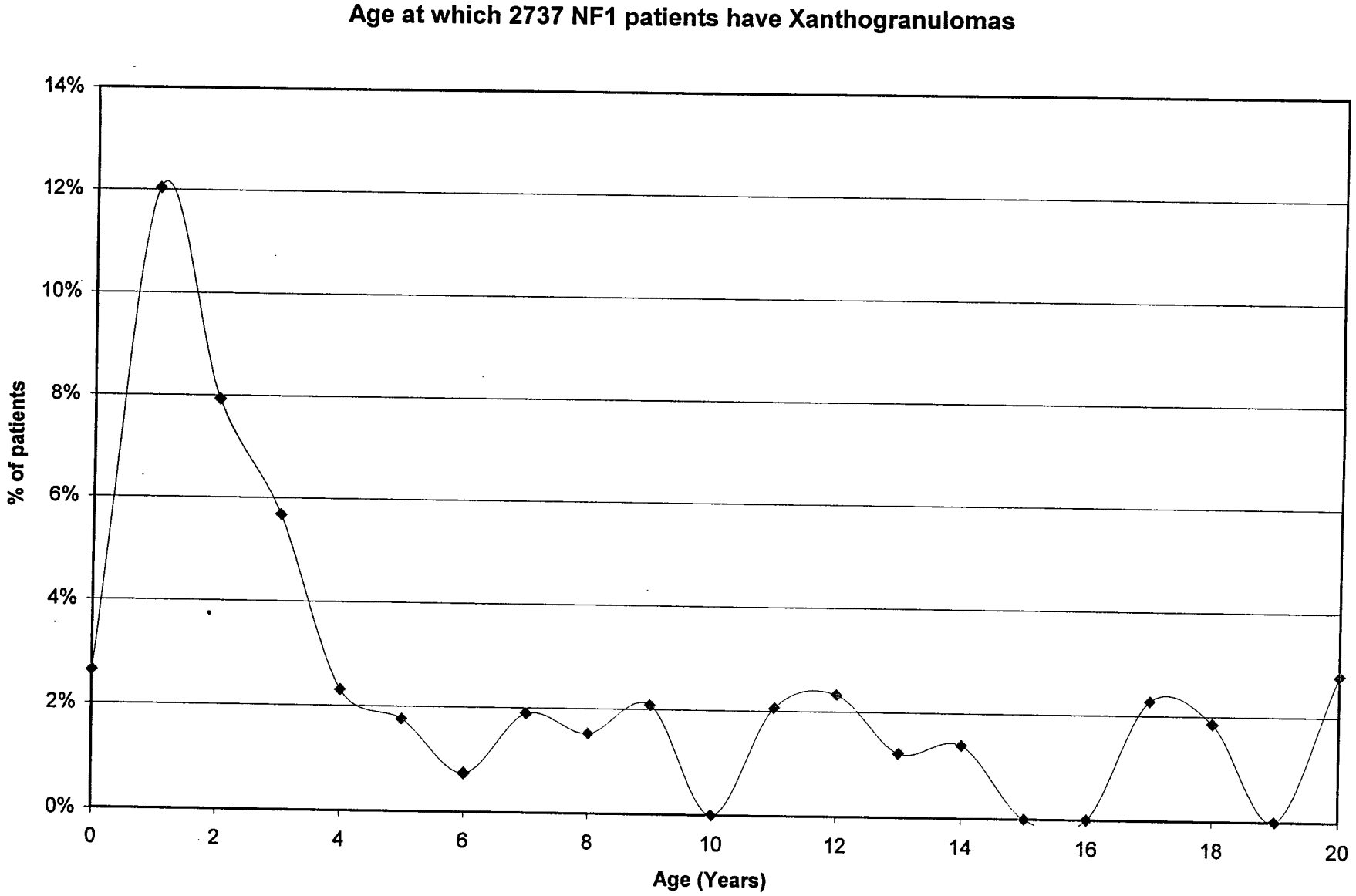
Appendix A – Sample graphs of feature prevalence by age

fig 1 - Lisch nodules
fig 2 - cutaneous neurofibromas
fig 3 - U.B.O.s
fig 4 - intertriginous freckling
fig 5 - café au lait macules
fig 6 - xanthogranulomas
fig 7 - late diagnosis group

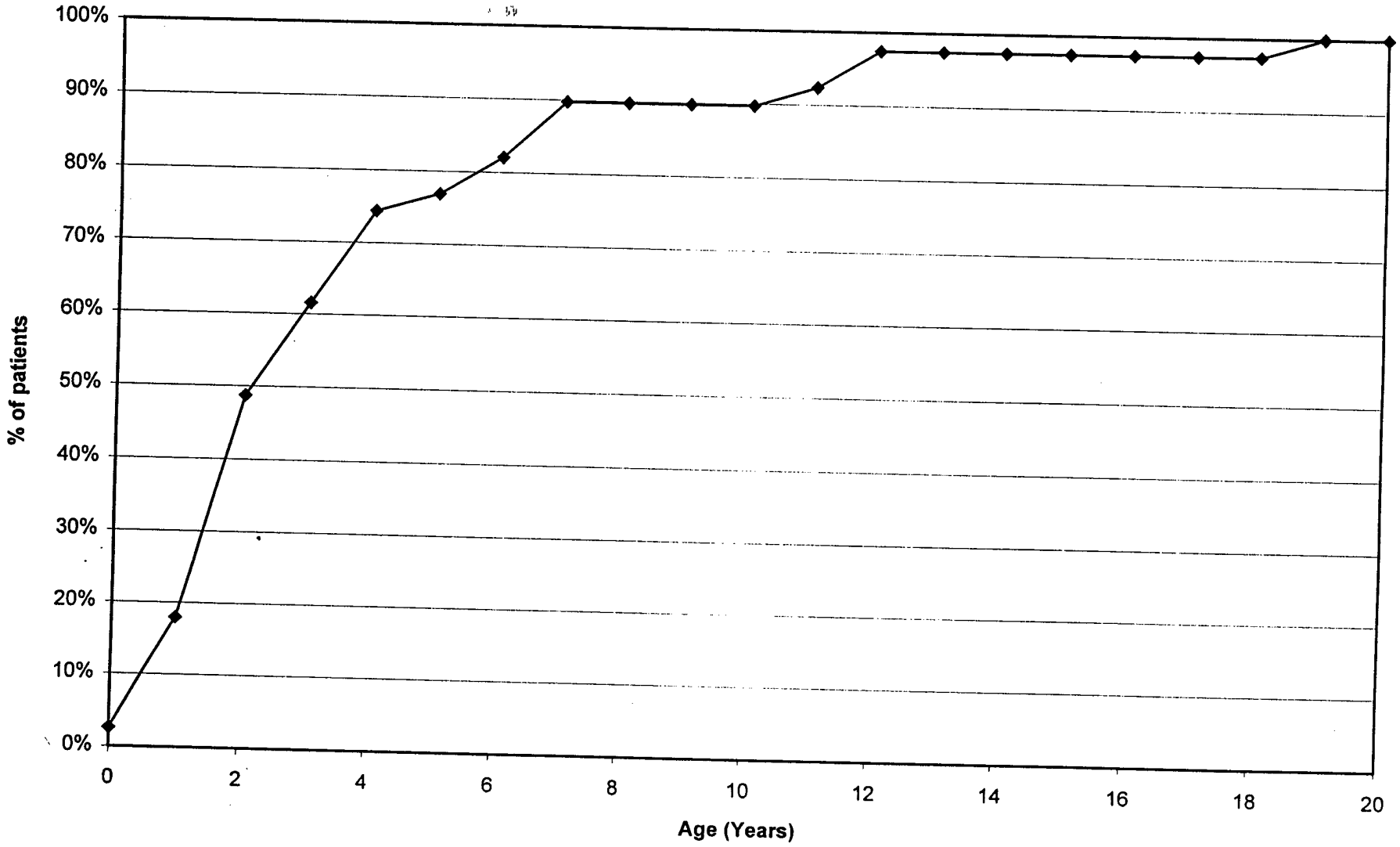

Age at which 870 NF1 patients have UBOs
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of patients
0
5
10
15
20
25
30
35
40
Age (Years)


Age at which 1835 NF1 patients have inguinal or axillary freckling
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of patients
0
2
4
6
8
10
12
14
16
18
20
Age (Years)


Age at which 1888 NF1 patients have cafe au lait spots
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% of patients
0
2
4
6
8
10
12
14
16
18
20
Age (Years)


Age at which 2737 NF1 patients have Xanthogranulomas
14%
12%
10%
8%
6%
4%
2%
0%
% of patients
0
2
4
6
8
10
12
14
16
18
20
Age (Years)

41 patients who met only one of the NIH diagnostic criteria for NF1 when initially evaluated.
All of these patients eventually developed other signs of neurofibromatosis and were diagnosed with NF1.



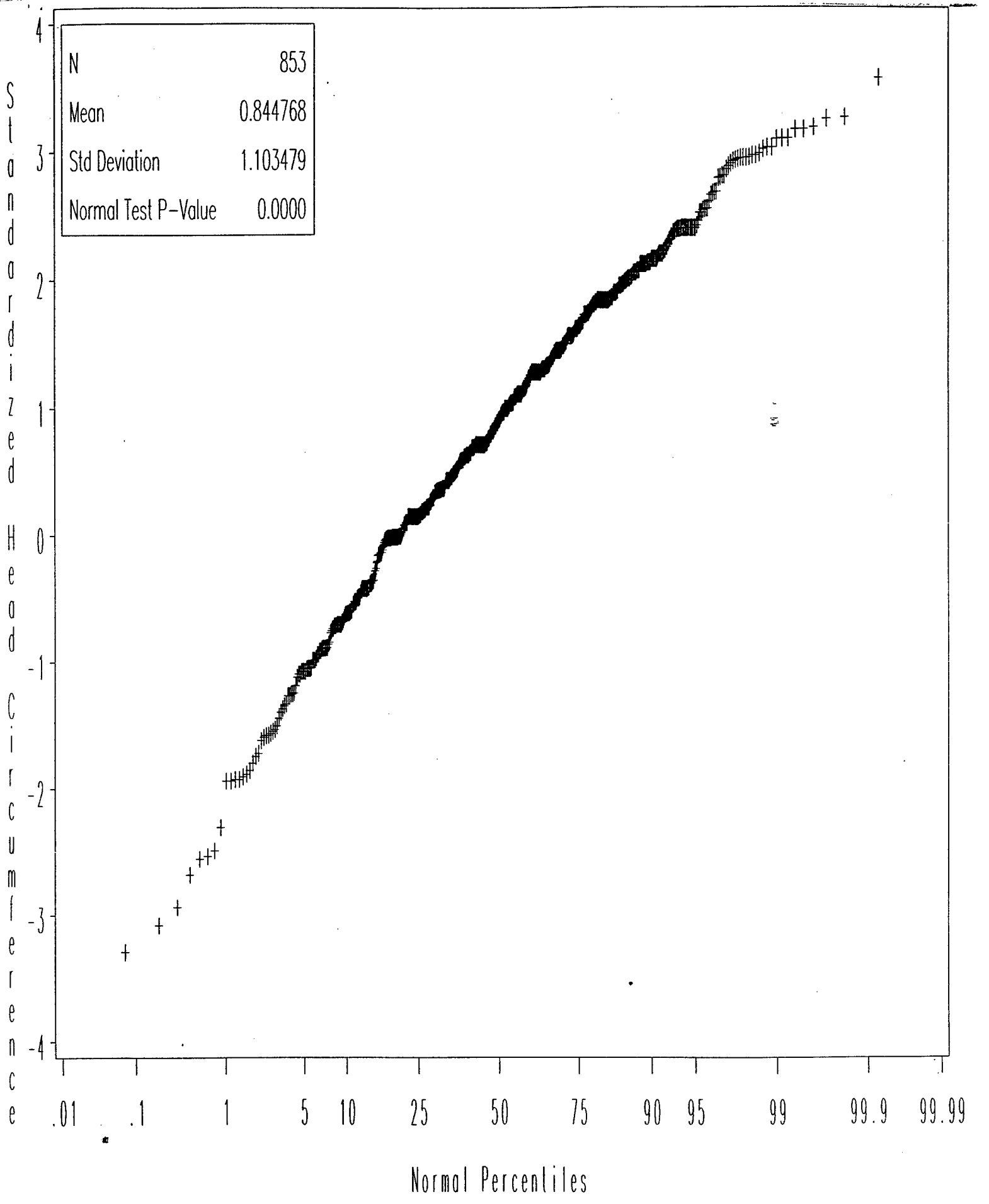
Appendix B – Sample 2 x 2 associations

fig 1 - occurrence of various neurofibromas
fig 2 - occurrence of freckling, cafe au lait and Lisch nodules
fig 3 - internal neurofibromas and hyperpigmentation

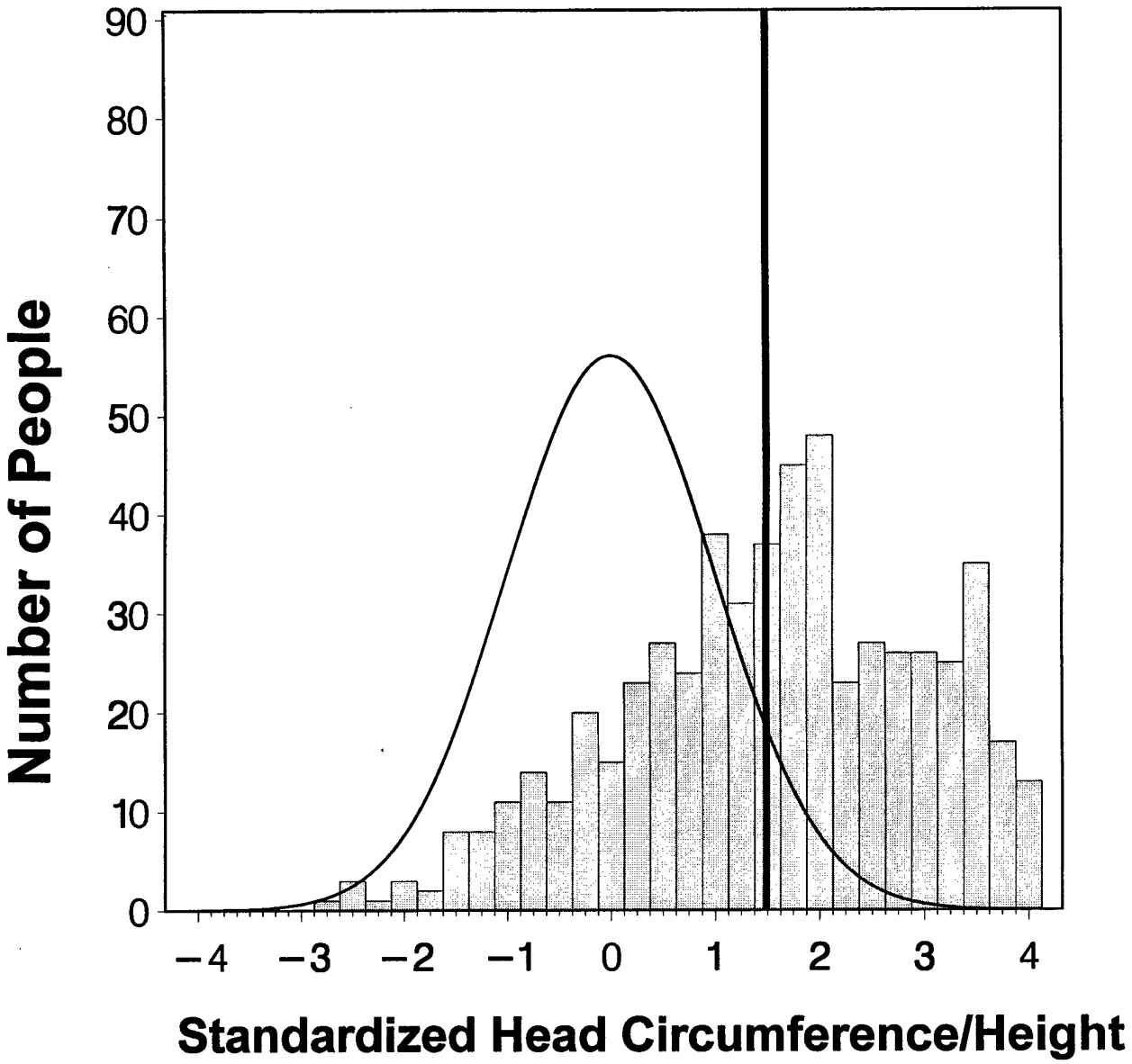
Appendix C – Sample growth charts

fig 1 - cumulative frequency distribution of head circumference
fig 2 - centile plot of head circumference
fig 3 - head circumference/height ratio by age
fig 4 - standardized head circumference/height measurements
fig 5 - centile plot of height by age
fig 6 - standardized heights measurements

Cumulative frequency distributions of head circumference z-scores, based on normal (unaffected) standards for 853 boys.



Head Circumference/Height in Males



Height in Males