NEUROFIBROMATOSIS TYPE 1:
A CLINICAL AND MOLECULAR GENETIC STUDY

Marjon H. Cnossen

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Neurofibromatosis type 1:
Een klinische en moleculair genetische studie

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To Neurofibromatosis patients and their families

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ABBREVIATIONS

Abbreviations

ASO	allele specific oligonucleotide analysis
CMC	chemical mismatch cleavage
CPP	central precocious puberty
CT	computed tomography
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleicacid
ES	embryonic stem
EVI2A/ EVI2B	human homologues of murine genes which cause murine leukaemia
FISH	fluorescence *in situ* hybridization
FPTI	farnesyl protein transferase inhibitors
GAP	GTPase activating protein
GHD	growth hormone deficiency
GM-CSF	granulocyte/ macrophage-colony stimulating factor
GTP	guanosine triphosphate
GDP	guanosine diphosphate
HA	heteroduplex analysis
JCML	juvenile chronic myeloid leukemia
MRI	magnetic resonance imaging
MS	multiple sclerosis
NIH	National Institutes of Health
NF	Neurofibromatosis
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
NF5	segmental Neurofibromatosis
NF6	familial *café-au-lait* spots
NF1-GRD	NF1 GAP-related domain
NF-NS	Neurofibromatosis-Noonan syndrome
NS	Noonan syndrome
OMGP	oligodendrocyte-myelin glycoprotein
PFGE	pulsed field gel electrophoresis
PDGF	platelet derived growth factor
PTT	protein truncation test
RNA	ribonucleicacid
RT-PCR	reverse transcribe polymerase chain reaction
SSCP	single strand conformational analysis
TGF-beta 1	transforming growth factor beta 1
TGGE	temperature gradient gel electrophoresis
VEP	visual evoked potential
WS	Watson syndrome
YAC	yeast artificial chromosome

Chapter 1

Introduction

1. Neurofibromatosis type 1
2. The *NF1* gene
3. Outline and aims of the study

1.1 A historical perspective

Neurofibromatosis type 1 (NF1), formerly known as von Recklinghausen's disease, has only been clinically defined since 1988, when the National Institutes of Health (NIH) Consensus Conference established the diagnostic criteria for the disease (1). However, clinical descriptions of possible NF1-patients date from as early as the 13th century (2). Symptoms described and depicted include areas of hyperpigmentation and skin tumors. In the *Anatomie Pathologique du Corps Humain* by Jean Cruveillhier (1791-1874) (3), anatomical studies clearly portrayed NF1 with skin tumors originating from the peripheral nerves. In 1882, Friedrich Daniel von Reckling-hausen published *Ueber die multiplen Fibrome der Haut und ihre Beziehung zu den multiplen Neuromen* (4), at the occasion of the 25th anniversary of Virchow's Pathology Institute in Berlin. Two patients with hyperpigmentation and skin tumors were described and the tumors consisting of neural elements and connective tissue cells became named neurofibromas. The classical studies of Crowe, Schull and Neel (5), established that *café-au-lait* spots are the first clinical manifestation of the disease and that six or more of these spots are pathognomic for the disease. Also axillary freckling became known as a hallmark of NF1 (6). In 1918, Waardenburg, the Dutch ophthalmologist, described the specific lesions of the iris seen in NF1, we now know as Lisch nodules (7). After Lisch (1937) concluded that these iris hamartomas were specific for NF1, his name became associated with this major disease feature (8). Their diagnostic significance was confirmed by Lewis and Riccardi and Toonstra, approximately 50 years later (9, 10). Familial occurrence of NF1 was reported by Akenside as early as 1768 (11) but the autosomal dominant inheritance of the disease was described by Preiser and Davenport (1918) (12). Later, studies by Borberg (13) and Crowe, Schull and Neel (5) confirmed the mode of inheritance and established a high penetrance of the disease and a high incidence of sporadic cases due to de novo mutations.

In 1987, the NF1 locus was mapped to the long arm of chromosome 17, near the centromere. Subsequently, cytogeneticists detected two unrelated NF1-patients with translocations involving 17q. One of the patients had a balanced translocation between chromosomes 1 and 17, t(1;17)(p34.3;q11.2). The other carried a balanced translocation between chromosomes 17 and 22, t(17;22)(q11.2;q11.2) (14, 15). These translocations were extremely helpful in the efforts towards gene identification by the International Consortium for Neurofibromatosis type 1 in 1990 (16-18).

1.2   Recognizing NF1 - the diagnostic criteria

Today, NF1 is an autosomal dominant disease, defined by diagnostic criteria with a wide variability of clinical manifestations, many causing considerable morbidity and even mortality in some cases. Approximately half of all NF1-patients represent de novo cases (19).

The diagnostic criteria of NF1 as established by the NIH (table 1.1.1)(1), include the following **major disease features** which are predominantly of neural crest origin: *café-au-lait* spots, freckling in the axillary or inguinal region, neurofibromas, Lisch nodules, optic pathway glioma, distinctive osseous lesions (sphenoid dysplasia, thinning of long bone cortex with or without bowing and pseudoarthrosis) and also a positive family history of NF1. Two or more criteria are necessary for a diagnosis of NF1. It is noteworthy that an affected first degree relative is considered equally relevant for the diagnosis as one of the major disease features. Most major disease features appear with increasing age. Nevertheless, diagnosis should be possible by the age of 5 years as the penetrance of the disease is considered virtually 100% at this age (19).

Table 1.1.1

Diagnostic criteria for Neurofibromatosis type 1 (NF1) (1)

2 or more criteria are necessary for a diagnosis of NF1

Major disease features

- 6 or more *café-au-lait* spots over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals.
- Two or more neurofibromas of any type or one or more plexiform neurofibromas.
- Freckling in the axillary or inguinal region.
- Two or more Lisch nodules (iris hamartomas).
- Optic or chiasma glioma.
- A distinctive osseous lesion, such as sphenoid dysplasia or thinning of long bone cortex, with or without bowing and pseudoarthrosis.

Family history

- A first-degree relative (parent, sibling or offspring) with NF1 according to the criteria above.

Café-au-lait spots are generally the first presenting symptom of NF1 and are observed in 82% of NF1-affected children before the end of the first year (figure 1.1.1) (20). In adults, *café-au-*

lait spots tend to become less pronounced and may even disappear (21, 22). *Skin fold freckling* in the axillae or groin is often the next presenting major disease feature and is observed in 81% of children before 6 years of age (figure 1.1.2) (23, 24). Skin fold freckling may also be encountered in the nape of the neck, under the chin and in the female submammary region (25).
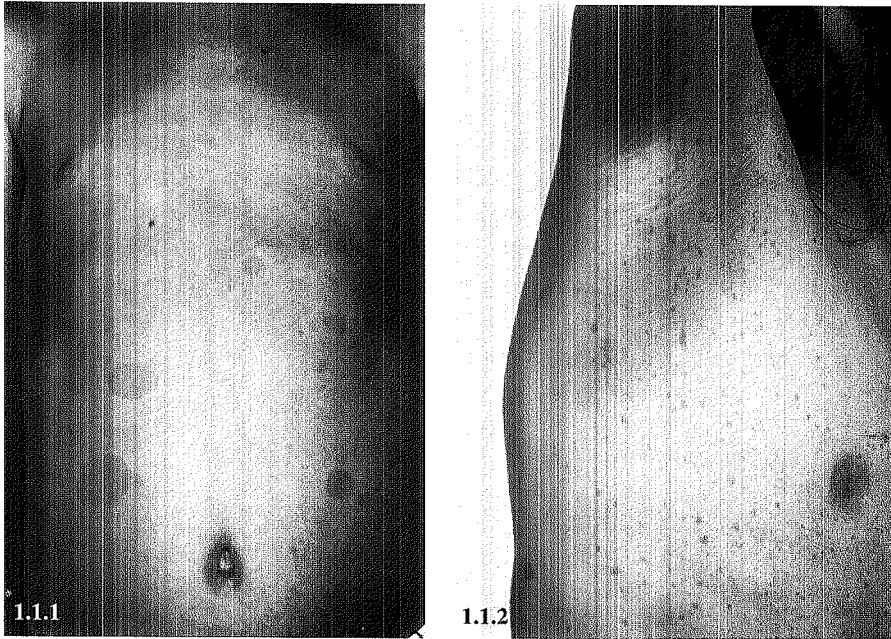
Figure 1.1.1
A child with café-au-lait spots. Typically they have smooth contours and may vary in diameter from 0.5 cm to 50 cm or more. Their color intensity varies with background skin pigmentation. Sometimes an ultraviolet (Woods) lamp may be necessary to assess their presence (courtesy Dr A.P. Oranje).

Figure 1.1.2
Skin fold freckling in the axillae. Freckling is often seen in other sites of skin apposition such as the groins, the nape of the neck, under the chin, in the female submammary region, folds between the upper eyelids, or folds resulting from obesity. Also they may present diffusely over the trunk and proximal extremities (courtesy of the late Dr P. Fleury).

Neurofibromas are divided into two major types: dermal and plexiform neurofibromas. Both types consist of a mixture of Schwann cells, perineural fibroblasts, endothelial cells and mast cells (26). *Dermal neurofibromas* are cutaneous or subcutaneous tumors, which originate from terminal nerve branches in the skin (figure 1.1.3). They start to appear prior to puberty and

mainly develop on the trunk. They are rarely painful but may cause pruritus (22). During adulthood, these benign tumors may become a major cosmetic burden. A large study on pregnancy outcome in NF1-patients reported growth of new neurofibromas in 60% of cases and enlargement of existing neurofibromas in 52% (27).
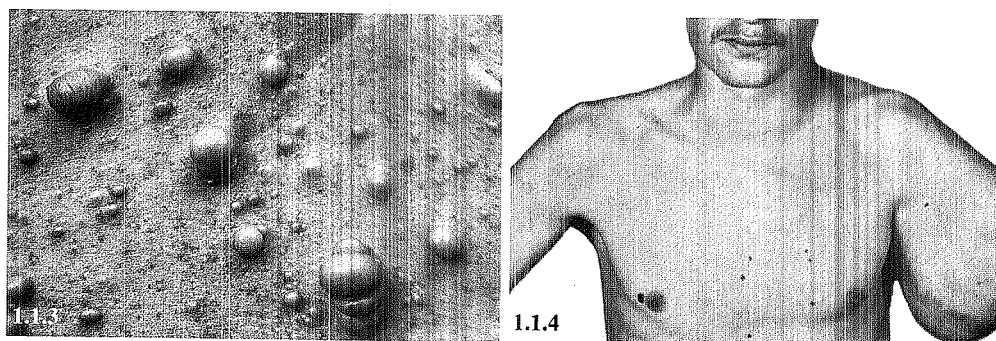
Figure 1.1.3
Dermal neurofibromas are either cutaneous or subcutaneous tumors. Cutaneous, they are characteristically reddish-bluish, soft, almost gelatinous to touch, and typically located on the trunk. Generally, they are sessile but may become pedunculated. Subcutaneous neurofibromas are discrete spherical or ovoid shaped and often firm to touch; they are more often painful or tender (courtesy of the late Dr P. Fleury).

Figure 1.1.4
A plexiform neurofibroma in the left axilla. These may present superficially as well as deeper within different tissues. Extension into adjacent tissues must always be suspected as particularly diffuse plexiform neurofibromas grow with numerous finger-like fronds (courtesy of the late Dr P. Fleury).

Plexiform neurofibromas originate from major nerve plexuses and major peripheral nerves (figure 1.1.4) and as a consequence may cause severe complications (see 1.2.1). They are present in approximately one third of the NF1 population and are congenital, although they may present later due to growth (22). The overlying skin may be abnormal with either signs of hypertrophy, hyperpigmentation or hypertrichosis. Two types of plexiform neurofibromas have been

described (28). Diffuse plexiform neurofibromas are soft subcutaneous swellings with ill defined margins. Nodular plexiform neurofibromas are ovoid- or spherically shaped, feel firm and are well circumscribed.

Lisch nodules are small pigmented hamartomas of the iris. They are present in 92% of NF1-patients older than 6 years of age (figure 1.1.5) (9). As a consequence, these iris hamartomas are of great diagnostic importance for NF1.
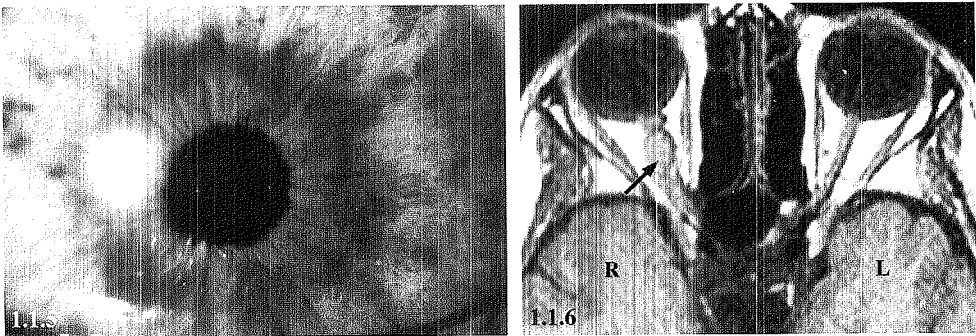
Figure 1.1.5
Lisch nodules are benign iris hamartomas and are classically present in both eyes. Slit lamp examination allows distinction from iris naevi. Lisch nodules present as three-dimensional translucent masses, punctuated by pigment cells (courtesy of the late Dr P. Fleury).

Figure 1.1.6
An optic pathway glioma of the right optic nerve (arrow) as best visualized by MRI-imaging rather than CT-scan (courtesy H. Stroink).

Optic pathway glioma or pilocytic astrocytoma of the optic pathway, are an important complication of NF1 in childhood (median age of presentation of symptomatic tumors: 4.9 years) (figure 1.1.6) (29). Generally, optic pathway glioma associated with NF1 are less progressive than in children without NF1. Symptomatic optic pathway gliomas seldomly appear after 6 years of age (29). Routine screening with MRI-imaging in 176 children showed optic pathway glioma in 33 (19%). However, only half of these children developed glioma-associated signs or symp-

toms; progression of tumor growth and deteriorating vision was observed in only 3 children (9%). The mean follow-up time in this study was 4.2 years (30).

Osseous lesions specific for NF1 are *sphenoid wing dysplasia*, and congenital *bowing or thinning of long cortical bones* with or without pseudoarthrosis (1). Sphenoid wing dysplasia (figure 1.1.7) is rare in NF1-patients but occasionally may present with pulsatile exophthalmus (31). Congenital bowing is generally anterolaterally and located in the tibia or fibula (figure 1.1.8), it is reported in 3% of NF1-patients (32). However, NF1 has been implicated in more than half of cases of congenital pseudoarthrosis of the tibial bone (33, 34).
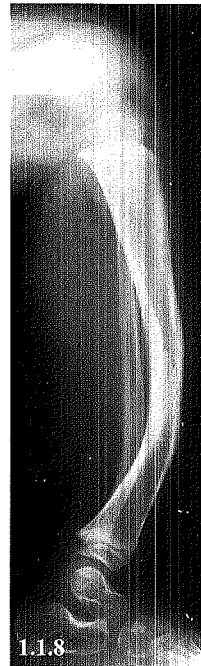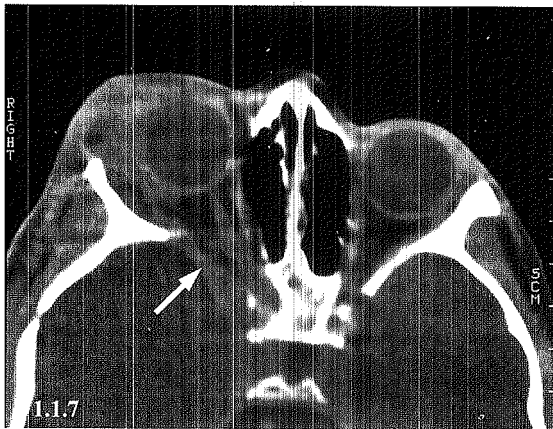
Figure 1.1.7
Sphenoid wing dysplasia *of the right ala major of the sphenoid bone (arrow). These defects are generally unilateral and not always associated with a plexiform neurofibroma. The great wing is affected more often than the small wing. An extensive disruption in the orbital wall may cause a pulsatile exophthalmos with or without herniation of the brain into the orbital space (courtesy H. Stroink and T.R Hendriksz).*

Figure 1.1.8
Congenital bowing *of the left tibial bone. Bowing with or without pseudoarthrosis is also seen in other long bones, but is rare (courtesy Dr A.M.F Diepstraten).*

1.3 Clinical manifestations

Complications in NF1 may be due to plexiform neurofibromas, optic pathway gliomas, skeletal abnormalities, severe scoliosis, hemihypertrophy, but also to mental retardation, epilepsy, endocrinological abnormalities, hypertension, and an increased risk of malignancies.

Also, the following **symptoms** are frequently observed in NF1-patients: macrocephaly, short stature, hypertelorism, thorax abnormalities, headache, mild scoliosis, and various learning-, speech-, motor- and behavioral disabilities. Macrocephaly, short stature, hypertelorism, thorax abnormalities and learning disabilities are so typical for NF1-patients that they are considered characteristic though **minor features** of the disease (28). As most symptoms and complications are age dependent, a chronological classification is used to describe the clinical manifestations in this section (20, 25, 35).

During infancy
Café-au-lait spots, plexiform neurofibromas, anterolateral bowing of the tibia (with or without pseudoarthrosis) and hemihypertrophy are generally congenital or present during the first year of life (see 1.2). Hemihypertrophy is not always associated with a plexiform neurofibroma.

Macrocephaly is often observed in NF1-patients. Headcircumferences larger than the 97th centile are observed in 45% of NF1-patients (21). In general, NF1-patients tend to be shorter than their unaffected siblings with a height at or below the fifth centile in one third of cases in one study (22). The cause for short stature is unknown. Hypertelorism is also frequently observed and contributes to the "facies Neurofibromatosis" as described by Lin (36). Further typical facial features are a broad nasal bridge or root, pigmentary abnormalities ("dirty skin") and facial asymmetries. In addition, thorax abnormalities, such as pectus excavatum or pectus carinatum, are often seen (22).

During early childhood
Freckling in the skin folds and optic pathway glioma are the diagnostic criteria becoming manifest during early childhood (see 1.2).

Learning disabilities are observed in 30%-45% of NF1-patients (37). Cognitive problems include a lower full scale IQ, multidimensional cognitive deficits, reading disabilities and neuromotor deficits (38, 39). Speech-, and behavioral problems are also frequently observed but are not specific (28). Speech- and motor problems may often require support by a speech therapist or

physical therapist. Recent brain imaging and neuropsychological assessment showed a significant association between lower IQ and T2-weighted hyperintensic regions on MRI imaging (38, 40), which was not confirmed by others (41-43). A severe complication of NF1 is mental retardation, although much less common than learning difficulties with prevalences between 4.8%-8% (40, 44).

On T2-weighted MRI imaging, increased-intensity lesions are seen in up to 79% of NF1-patients in the basal ganglia, thalamus, cerebellum and brainstem regions of the brain in NF1-patients. They present as well circumscribed, hyperintense foci, nonenhancing after administration of contrast medium, without mass effect and resolving with increasing age (average age of presentation: 7 years) (45). Histopathologically, the hyperintense lesions on T2-weighted MRI images correspond to areas of vacuolar or spongiotic change (47). These lesions should be carefully differentiated from brainstem tumors which cause focal or diffuse brainstem enlargement, mass effect and gadolinium enhancement. Generally, these brain stem tumors tend to grow less aggressively than in children without NF1 (46).

Precocious- and delayed puberty and growth hormone deficiency are more frequent in children with NF1 than in the general population (48). Therefore, deviations from the growth curve and premature manifestations of puberty should be recognized as possible complications of NF1. Although central precocious puberty in NF1 is said to be associated with an optic pathway (chiasma) glioma, our and one other study observed children with NF1 and central precocious puberty or growth hormone deficiency without optic pathway (chiasma) glioma (49, 50).

Hypertension in NF1-patients may be associated with either renal artery stenosis or pheochromocytoma (28). In children with NF1, pheochromocytoma is however quite rare. There is a slightly increased risk for epilepsy in NF1-patients (28), which is only partly accounted for by macroscopic abnormalities. It has been suggested that headache of various types (tension headaches, migraine) may be more common in NF1-patients than in the general population (51).

During late childhood and adolescence
Dermal neurofibromas begin to appear prior to puberty and may cause emotional distress. In addition, scoliosis typically occurs or progresses during puberty. Both idiopathic and dystrophic scoliosis is described. The dystrophic curve is short and sharp, involves four to six segments, distorts vertebral bodies and ribs and has a poor prognosis (52).

Lifelong

A descriptive analysis of NF1 in almost 1800 patients with an average age at examination in probands of 16.9 years (n = 1.479; range: 0-73 years) and of affected relatives of 22.2 years (n = 249; range: 0-80 years), observed malignancies (excluding optic pathway gliomas) in approximately 6.9% of probands and 4.4% of relatives (53). In addition to malignant peripheral nerve sheath tumors which may arise in preexisting plexiform neurofibromas, astrocytomas, juvenile chronic myeloid leukaemia (JCML) (54), rhabdomyosarcoma (20), adenocarcinoma of the ampulla Vater and certain duodenal tumors (55), have been reported to be associated with NF1. Moreover, secondary tumors are known to develop in 21% of NF1-patients with primary malignancies (56) in comparison to 4% in the general population (57).

The wide spectrum of learning and behavioral difficulties presenting in childhood in NF1-patients, may lead to social isolation, particularly in de novo cases. Although some studies report high prevalences of psychiatric illness (depression, anxiety) (58, 59) this has not been confirmed by others (28).

In summary, complications in NF1-patients may occur in every organ or tract and may cause severe morbidity and even mortality. The course of the disease is unpredictable as the variability is extreme, even between family members. Generally, one third of the patients have moderate to severe complications of the disease (51).

1.3.1   Management of NF1-patients

Major disease features

Major disease features needing careful follow-up by NF1 specialists are plexiform neurofibromas, optic pathway gliomas and problematic osseous lesions, such as bowing of the tibia.

Depending on their localization and symptomatology, plexiform neurofibromas may cause considerable morbidity such as functional disturbances, neurological deficits and cosmetic disfigurement. Threatening localizations include the head and neck, in the vertebral column, paravertebrally and the perineal region. Moreover, malignant peripheral nerve sheath tumors (synonyms: neurofibrosarcoma, malignant schwannoma) and the extremely rare Triton tumor (variant of malignant peripheral nerve sheath tumor, showing rhabdomyoblastic differentiation) (60), may arise within a plexiform neurofibroma. In those cases in which surgery is indicated,

complications as bleeding and recurrence of the tumor are well known problems (22). Generally, total excision of a plexiform neurofibroma is impossible due to fingerlike fronds that insinuate themselves into adjacent tissues. Excessive bleeding is caused by vascular abnormalities and the presence of mast cells within the plexiform neurofibromas (61). Although growth and appearance of dermal neurofibromas has been described during pregnancy, growth patterns of plexiform neurofibromas have not been studied (27).

Quite recently, a consensus was reached on the management of optic pathway glioma. The NF1 optic pathway glioma task force recommends annual ophthalmological examinations during the first decade of life (until 6 years of age) according to tables 1.1.2 and 1.1.3., by an experienced ophthalmologist, supplemented by visual evoked potentials and visual field evaluations (29). According to the consensus, complete ophthalmological examinations should include visual acuity, color vision, visual fields, eye movements, pupillary light responses, refractive status, funduscopy (indirect and direct) and slit lamp examination. Evidence of dysfunction of the optic nerve should be followed up by magnetic resonance imaging (MRI) of the brain with contrast enhancement and special attention to the orbits. Retrospectively, these guidelines were largely followed in our study. Management of children with optic pathway glioma depends on the localization of the tumor; chiasmatic tumors have been reported to progress more frequently and may be accompanied by hormonal disturbances (62). If clinical progression is observed, treatment may be indicated and may include chemotherapy (63) or less commonly radiation therapy or surgery (64, 65).

Table 1.1.2
Ophthalmological screening protocol
for the child with NF1 (29)

1.	All children below 6 years: once yearly complete examination [1]
2.	All newly diagnosed NF1-patients: complete examination.
3.	Follow-up schedule of children over 6 years of age, in a multidisciplinary setting:
	Age 8, 13, 20 years: short examination [2]
	Age 10, 16, 25 years: complete examination
4.	Complete ophthalmological examinations should be performed whenever signs or symptoms suggest an ophthalmological abnormality

[1] Complete examination: visual acuity, color vision, visual fields, eye movements, pupillary light responses, refractive status, funduscopy (indirect and direct) and slit lamp examination.

[2] Short examination: visual acuity, color vision and slit lamp examination.

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Table 1.1.3
*Ophthalmological follow-up protocol of children
with optic pathway glioma (29)*

Time interval following diagnosis	Ophthalmological examination [1]	MRI
1st year	3 monthly	6 monthly
2nd year	6 monthly	6 monthly
Thereafter [2]	yearly	yearly

[1] Interval visual field examinations should be performed when the child is old enough to permit reliable examinations.

[2] If there is no evidence of progression 2 years after diagnosis, intervals can be gradually increased.

Children with congenital bowing must be monitored regularly by an orthopedic surgeon with experience in NF1 as prevention of bone fractures and pseudoarthrosis is essential. Treatment may consist of prophylactic braces and/ or surgery by bone grafting.

Other symptoms and complications
After underlying causes such as neurofibromas are ruled out (25), remaining symptoms and complications are generally treated as in patients without NF1.

High percentages of spontaneous abortion, preeclampsia, intrauterine growth retardation, preterm delivery and perinatal mortality have been reported in pregnancies of women with NF1 (66, 67). However, a recent study showed only a higher percentage of caesarean sections. It was concluded that there may be a subgroup of NF1-affected women who may be at risk for obstetrical complications but that the overall frequency is not higher than in the general population. However, risk factors for obstetrical complications were not given (27).

Genetic counseling
Information on the inheritance of NF1, its natural history and the possibilities and limitations of molecular genetic analyses are essential for patients and family members. In NF1 and other diseases, significant differences have been reported in informedness of patients who received genetic counseling and those who had not (68). Therefore, consultation of a clinical genetic center is recommended.

1.4   Phenotypical variation

Apart from the variability in clinical manifestations observed in NF1-patients a number of distinct **subphenotypes** have been described, such as **Neurofibromatosis-Noonan syndrome (NF-NS)** and **Watson syndrome (WS)**.

Patients with **NF-NS** exhibit both features of NF1 and typical symptoms of Noonan syndrome (NS) such as short stature, developmental delay, facial anomalies and congenital heart defects (generally valvular pulmonary stenosis). Prevalences of NF-NS have been reported of 6.4% (69). **WS** patients are characterized by short stature, cognitive impairment and pulmonary stenosis (70, 71).

There have been longstanding discussions if NF-NS and WS are either variants of NF1, a result of a contiguous gene syndrome or only chance associations between NF1 and both syndromes. In two families clinical and molecular genetic evidence was found that the occurrence of NF1, NS and WS are caused by the same NF1 allele. After diagnosis of WS in the first family, an 80 kb deletion was detected in the NF1 gene (72). In a second family with features of NF1, NS and WS, an in-frame tandem duplication of 42 bases was found in exon 28 of the NF1 gene (73). Furthermore, cosegregation of markers flanking the NF1 gene has been reported in patients with NS features (74).

This latter evidence however, is also suggestive of a contiguous gene syndrome. Several NF1 gene deletions >700 kb have been observed in patients with Noonan-like features (75-80). In addition, recently, a large kindred was reported with both NF1 and NS. Linkage analysis in this pedigree demonstrated that the NF-NS phenotype was caused by two genetically linked but distinct mutations (81) and was therefore suggestive for a locus for NS in the vicinity of the NF1 gene.

In contrast, several studies in NS patients using intragenic and extragenic polymorphic markers show lack of linkage to the NF1 locus (82-84). Moreover, in NS linkage has been established to chromosome 12q in a small number of families (85).

In summary, WS definitely seems to be a variant of NF1. On the contrary, the clinical variability of NF1, the phenotypical overlap with NS and NS locus heterogeneity, makes conclusions on whether NF-NS is an allelic variant of NF1, due to a contiguous gene syndrome or a chance association difficult. Conceivably, NF-NS may not have an identical genetic background in each pedigree.

1.5   Prevalence, life expectancy and genetic fitness

Crowe et al. (5) reported a first prevalence figure of NF1 of 1/2500 - 1/3300. Later studies obtained similar findings (19, 58, 86, 87). No geographical or ethnic differences for the prevalence of the disease have been observed. Differences in birth incidence and prevalence according to age suggest that NF1-patients have a reduced life span.

Sorensen et al. (56) established survival rates in NF1-patients after 40 years follow-up of a group of NF1-patients recruited by Borberg in 1944 (13). This cohort consisted of 84 probands traced through hospital records and 128 NF1-affected family members. Follow-up rates were 99%. Bias towards severity was limited by determining only mortality rates in the NF1-affected family members. Survival rate curves indicated some reduction in the life span of the NF1-affected family members as compared to the general population without giving specific numbers. The most common causes of death in this group did not differ from the general population. However, certain tumor types were more frequent in NF1-affected family members than in the general population.

Genetic fitness is graded from 0 to 1 and indicates the ability to procreate. Failure to reach the reproductive age, excessive spontaneous abortions, decreased fertility and difficulties in establishing permanent relationships may lead to a decrease in genetic fitness. In NF1, estimates for genetic fitness vary from 0.47 to 0.80 (5, 19, 88). Crowe et al. reported a significant difference between men and women of respectively 0.62 and 0.88 (5). Most likely causes for this reduction in genetic fitness seem premature death and social isolation, which may be more pronounced in men than women. Possibly, a slight decrease in male fertility may also play a role.

1.6   Classification - other Neurofibromatoses

In 1981, Riccardi suggested a subdivision into seven different types of Neurofibromatosis (NF) (51). These types differed in occurrence, number and distribution of major disease features (table 1.1.4). Momentarily, only NF1, NF2 and NF5 are used to classify NF-patients as the remaining subtypes are ill-defined or rare, making their clinical use difficult (28). There is some evidence that NF6 or familial *café-au-lait* spots may also be a form of NF. Several families with multiple *café-au-lait* spots have been reported. Although linkage to 17q was found in one family (89), this could not be confirmed in 2 other families (90, 91).

Table 1.1.4

Neurofibromatosis types according to Riccardi (1981)

	Synonyms	Definition
NF1	von Recklinghausen's disease Peripheral Neurofibromatosis	see table 1.1.1
NF2	Central Neurofibromatosis	see table 1.1.5
NF3	mixed type of NF	mixture of key features NF1 and NF2 no Lisch nodules no optic pathway glioma no short stature, no learning disabilities, no pectus excavatum
NF4	variant NF	NF phenotypes not fitting into NF1, NF2 or NF3
NF5	segmental NF	localized NF
NF6	*café-au-lait* spots only	*café-au-lait* spots with sometimes minor disease features
NF7	late onset NF	major disease features presenting in the third decade or later
NF-NOS	includes: gastrointestinal NF multiple schwannomatosis familial brain tumors	neurofibromas limited to the gastrointestinal tract multiple schwannomas in skin and/ or spinal cord familial brain tumors of multiple types

In 1988, the NIH Consensus Conference also established diagnostic criteria for Neurofibromatosis type 2 (NF2) (1). Recently, the criteria for NF2 have been slightly adapted (table 1.1.5) (25). Until the diagnostic criteria were formulated, many studies had failed to clinically distinguish NF1 from NF2 as there were a number of overlapping symptoms (*café-au-lait* spots, neurofibromas). Later on, the identification of both the NF1 gene on chromosome 17q11.2 (16-18) and the NF2 gene on chromosome 22q12 (92, 93) proved that these were two distinct genetic entities.

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Table 1.1.5
Diagnostic criteria for Neurofibromatosis type 2 (NF2) (25)

Individuals with the following clinical features have confirmed (definite) NF2:

1.	Bilateral vestibular schwannomas (VS) **OR**
2.	Family history of NF2 (first degree relative) **PLUS**:
a.	Unilateral VS < 30 years **OR**
b.	Any 2 of the following:
	meningioma, glioma, schwannoma, juvenile posterior subcapsular lenticular opacities/ juvenile cortical cataract

Individuals with the following clinical features should be evaluated for NF2 (presumptive or probable NF2):

1.	Unilateral VS < 30 years **PLUS** at least one of the following: meningioma, glioma, schwannoma, juvenile posterior subcapsular lenticular opacities/ juvenile cortical cataract
2.	Multiple meningiomas (2 or more) plus unilateral VS < 30 years **OR** one of the following: glioma, schwannoma, juvenile posterior subcapsular lenticular opacities/ juvenile cortical cataract

Definitions introduced for NF5 or segmental NF differ greatly. Riccardi defined segmental NF as *café-au-lait* spots (and/or freckling) accompanying neurofibroma(s) to the side of the body ipsilateral to the tumor(s), with no crossing of the midline. It was described as non-familial and without systemic involvement (22). Roth based his classification on the estimated timing of the NF1 gene mutation during embryonal development and divided NF5-patients into four subsets: true segmental (non-inherited, segmental café-au-lait spots and/or neurofibromas, without systemic involvement), localized cases with deep involvement, inherited segmental Neurofibromatosis and bilateral segmental Neurofibromatosis (94).

2.1   The *NF1* gene and its mutability

Positional cloning techniques mapped the *NF1* gene to chromosome 17q11.2. It spans a region of about 350 kb of genomic DNA (16-18) and contains approximately 60 exons (figure 1.2.1 and table 1.2.1). (95). The *NF1* gene encodes an 11-13 kb mRNA which is markedly expressed in neurons, oligodendrocytes and nonmyelinating Schwann cells (96). Three genes: *EVI2A*, *EVI2B* and the gene encoding oligodendrocyte-myelin glycoprotein (*OMGP*), have been detected within

intron 27 (figure 1.2.1) (97, 98). *EVI2A* and *EVI2B* are human homologues of murine genes (murine chromosome 11) postulated to cause murine leukaemia after activation by retroviral insertion. *OMGP* encodes a protein expressed in myelinating Schwann cells and oligodendrocytes. Interestingly, all three genes are transcribed in opposite direction with respect to the *NF1* gene. NF1 homologous loci have been reported at additional chromosome locations (chromosomes: 2, 12, 14, 15, 20, 21 and 22). Most loci represent nonprocessed pseudogenes. However, two regions on chromosome 12 have open reading frames.
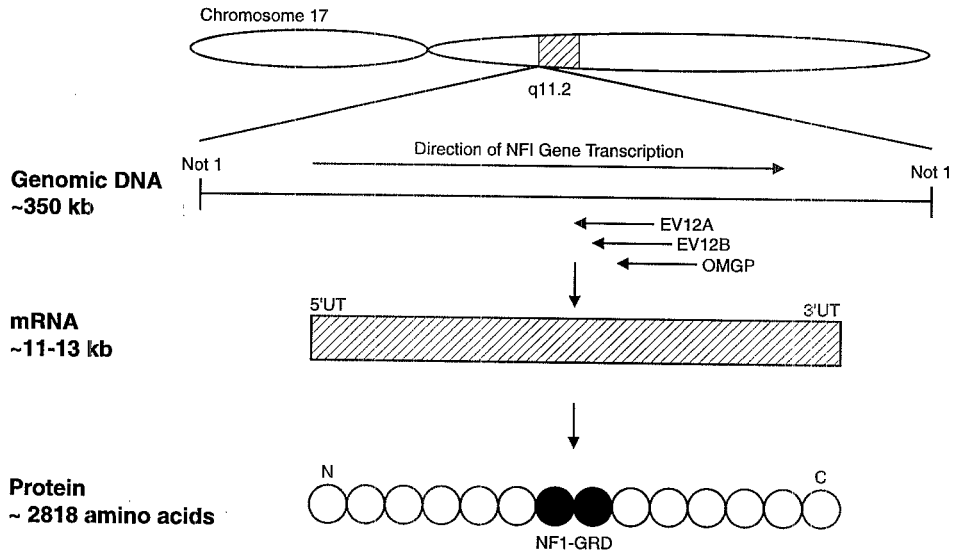
Figure 1.2.1
The NF1 gene location and size, with its embedded genes. The NF1 gene transcript and protein with its only known functional domain NF1-GRD.

The mutation rate of the *NF1* gene (1 x 10^{-4} per gamete per generation) is one of the highest among human disease genes (19) and cannot be explained by the large size of the gene alone. Mutations are spread throughout the gene and are of different types and sizes. Most unrelated patients have their own unique mutation. However, a number of recurrent mutations have been reported. The first and best known, in exon 31, R1947X (5890 C->T), was detected in at least 11 out of approximately 458 patients (2.4%), 10 of which have been published (16, 99-103). A nonsense mutation in exon 37 (C6792A) has been reported in 2 out of 92 unrelated patients (2.1%) (104). In total, 6 mutations have been found within a 4 base pair sequence in exon 37 (2 x C6792A, 2 x 6789delTTAC, 1 x 6790insTT, 1 x 6791insA + 5 base pair deletion

Table 1.2.1
Coding exons of NF1 gene (95)

Exon no.	cDNA position	Size (bp)	Introns (kb)	Exon no.	cDNA position	Size (bp)	Introns (kb)
1	1	60	?(20-140)	(23a	4111	63	6.0)
2	61	144	3.10	24	4111	159	0.53*
3	205	84	?	25	4270	98	1.25
4a	289	195	?	26	4368	147	1.27
4b	484	103	2.0	27a	4515	147	?
4c	587	68	0.22*	27b	4662	111	45-50
5	655	76	0.80	28	4773	433	1.3
6	731	158	?	29	5206	341	2.7
7	889	174	0.40	30	5547	203	4.3
8	1063	123	?	31	5750	194	1.55
9	1186	75	4.0	32	5944	141	0.15
10a	1261	142	?	33	6085	280	0.40
10b	1393	135	4.0	34	6365	215	0.24
10c	1528	114	2.5	35	6580	62	0.15
11	1642	80	0.54	36	6642	115	0.57
12a	1722	124	?	37	6757	102	1.7
12b	1846	156	1.2	38	6859	141	2.4
13	2002	250	0.49	39	7000	127	6.0
14	2252	74	0.23	40	7127	132	0.93
15	2326	84	1.3	41	7259	136	2.0
16	2410	441	0.38*	42	7395	158	4.0
17	2851	140	0.28*	43	7553	123	0.35
18	2991	123	0.46*	44	7676	131	0.18
19a	3114	84	1.2	45	7807	101	1.1
19b	3198	117	0.55	46	7908	143	0.35
20	3315	182	0.12*	47	8051	47	1.4
21	3497	212	2.2	48	8098	217	6.5
22	3709	162	0.14	(48a	8315	54	6.7)
23.1	3871	104	?	49	8315	153	
23.2	3975	136	4.0		- 3' Noncoding -		

Intron lengths were determined either by PCR amplification using facing exon primers from adjacent exons or by complete sequence between exons (denoted by an asterisk). Question marks denote inability to amplify by PCR across the intron; such introns are likely to be larger than 4 kb.

20 nucleotides downstream) in 6 unrelated patients out of more than 116 patients (105). Therefore, this region in exon 37 may also be considered a mutational "hot spot". In addition, a small deletion in exon 17 (2970delAAT) and an insertion (5451insC) in exon 29 were each detected twice in 150 unrelated cases (72). Upadhyaya et al. (106) and Li et al. (107) reported the missense mutation K1423E (4267 A->G) in a total of 5 unrelated patients. In addition, deletions encompassing the entire *NF1* gene have been detected in at least 26 patients (108). However, the boundaries of these deletions have not been established in all cases.

Different biological mechanisms may account for the high mutation rate. Firstly, the *NF1* pseudogenes located on multiple chromosomes may form a reservoir of mutations that can be crossed into the *NF1* gene by interchromosomal gene conversion (95, 109, 110). Secondly, the high mutation rate of the *NF1* gene could be due to a high frequency of methylatable CpG residues within the *NF1* gene (111). DNA methylation of CpG dinucleotides has long been considered as a major contributor of point mutations leading to human disease, since it may precede deamination of 5-methylcytosine (112). In one study, three exons (exon 28, 29 and 31) were examined for the presence of methylated sites. Nearly all methylation occurred at cytosines located within CpG dinucleotides (111). Lastly, particular sequence patterns in the *NF1* gene such as direct repeats, palindromes, quasi-palindromes, symmetrical elements and runs of identical bases may predispose towards insertions and/ or deletions.

Initially, various studies highlighted that the majority (up to 90%) of de novo mutations in the *NF1* gene arise in the paternal allele (113, 114). Recently, a collaborative study in 470 individuals with a de novo mutation using intra- and extragenic markers, allowed analysis of the parent of origin in 33 families. Small mutations were of paternal origin in 9 out of 11 and of maternal origin in 2 of 11 families. Large deletions were of maternal origin in 16 out of 21 and of paternal origin in 5 out of 21 de novo cases (115). No birth order or parental age effects were reported in any of these studies (19, 113-115). Sex differences in mutational mechanism may be due to differences between the development of gametes in males and females. In males, both the greater mitotic activity and the methylation status of spermatozoa may predispose towards smaller mutations. Studies of sperm DNA showed CpG methylation to be a consistent factor within the *NF1* gene and thus a possible template for germline mutations (111). Deletions are thought to occur more often in the maternal DNA due to an increased frequency of recombinational error during female meiosis (116, 117).

In conclusion, the high mutation rate in the *NF1* gene and its paucity of mutational "hot spots" inevitably lead to distinct mutations in almost every NF1 family. The nonsense mutation R1947X, a 4 base pair sequence within exon 37 and the missense mutation K1423E form

exceptions as they have been found in several unrelated families. The mutability at these respective sites may be explained by the presence of cytosine methylation and specific sequence patterns. Conceivably, not all mutations in the *NF1* gene can be explained by the mechanisms described above.

2.1.1   Germline and somatic mosaicism

The high mutation rate suggests that mosaicism may occur more often in NF1 than in most other genetic diseases. Genetic mosaicism is a condition in which genetically distinct cell populations are observed within one individual. Mutations during early embryonic development, before the determination of the germline, will cause gonosomic mosaicism (affecting somatic tissues and the germline). Mutations occurring later may affect either the germ cells alone (germline mosaicism) or the somatic cells alone (somatic mosaicism) (figure 1.2.2) (118). This phenomenon has important implications for mutation analysis and genetic counseling in NF1 and other genetic diseases (119).

Recently, germline mosaicism was reported in a clinically normal father with two NF1-affected children (120). In approximately 10% of the father's spermatozoa, a 12 kb deletion was found encompassing exons 32 to 39. As the mutation was not detected in the DNA from his lymphocytes it was presumed that the mutational event occurred in a germ-cell precursor.

For a long time, segmental NF (NF5) has been viewed as a hallmark of somatic mosaicism: a postzygotic mutation in a single precursor cell for a large skin area as an explanation for the clinical findings. Reports of patients with an apparent segmental NF having children with nonsegmental NF1 are consistent with this hypothesis (121, 122). However, at the molecular level, the proof of this hypothesis still has to be provided, as no identifiable *NF1* mutations have been reported in segmentally affected cases.

Moreover, molecular proof of somatic mosaicism was found in three patients with generalized rather than segmental NF1. All patients were de novo cases. Interestingly, 2 of these 3 cases showed a late onset of *café-au-lait* spots, freckling and neurofibromas and no severe complications of the disease (123, 124). Contrastingly, the third patient was diagnosed at 7 months of age with *café-au-lait* spots and a large cervical plexiform neurofibroma (77). Presumably, variations in phenotype will depend on the proportion and distribution of cells carrying the mutation. Genetic mosaicism would provide an explanation for the phenotypical

variation between de novo cases with a mild, (sometimes incomplete) phenotype whose offspring are more severely affected. Examples of clinical variability due to somatic mosaicism have been reported for other diseases such as NF2 (125) and tuberous sclerosis (126).
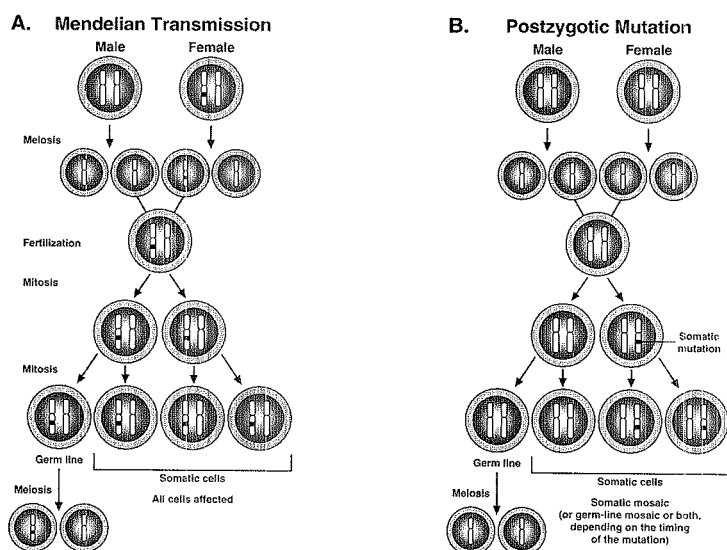
Figure 1.2.2
Mendelian transmission compared with postzygotic mutation
*Figure 1.2.2 A) shows the mendelian inheritance of a mutant gene through 2 generations. The mutation is present in all somatic and germline cells and is passed on with a possibility of 50% to the offspring. Figure 1.2.2 B) shows a somatic mutation arising after fertilization (postzygotic) causing a **genetic mosaicism**: only part of the individual's cells carry the mutation. Whether the mutant gene is passed on to the next generation depends on the timing of the mutation i.e. its presence in the germline.*

2.2 A tumor suppressor gene

In 1971, Knudson proposed his "second-hit model" for tumor development in retinoblastoma (127). According to this hypothesis, tumor formation is caused by complete inactivation of the tumor suppressing function of the retinoblastoma gene by mutations in both alleles. Hereditary retinoblastoma patients are predisposed to bilateral and multifocal tumors as they carry a germline retinoblastoma mutation in all somatic cells and only one secondary, postzygotic mutation is necessary for tumor formation (figure 1.2.3). Various studies have shown that this theory is also applicable to tumor formation in other tumor syndromes such as NF1, NF2, tuberous sclerosis and von Hippel-Lindau's disease (128). In *NF1*, loss or mutation of the wild

type allele has been detected both in malignancies (129-131) as well as in benign tumors (132, 133). Not necessarily all cells are affected within for example a neurofibroma. Loss of heterozygosity of the *NF1* gene has also been detected in tumors unassociated with the disease such as malignant melanoma, colonic carcinoma and neuroblastoma (107, 134, 135).

Schwann cell
in NF1-patient
(constitutional mutation)

Somatic mutation
of wild type allele

Neurofibroma or
Malignant peripheral
nerve sheath tumor
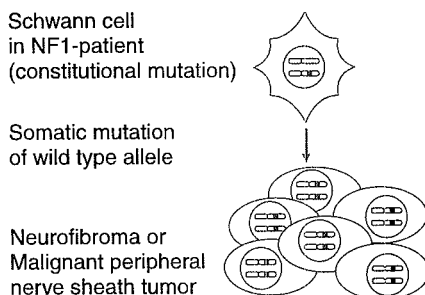
Figure 1.2.3
Knudson's "second hit" hypothesis applied to NF1. In a Schwann cell derived from an NF1-patient, one of the NF1 gene copies is constitutionally mutated (bar). A somatic mutation in the remaining wild type copy will result in complete loss of neurofibromin-mediated tumor suppression (2 bars) and therefore to tumor formation (benign: neurofibroma, malignant: malignant peripheral nerve sheath tumor).

Somatic mutation of the wild type *NF1* allele may also be involved in other symptoms and complications than tumor formation. In these cases, other domains (still unknown) of the *NF1* gene than the *NF1* GAP-related domain (*NF1*-GRD) (see 2.3) may be involved. Besides a "recessive" model in which a "second hit" is necessary, it seems plausible that insufficient levels of gene product due to the presence of only one normal copy of a gene (haploinsufficiency), may play a role in the development of NF1-related symptoms. The clinical expression of the disease may also depend on the tissue specific localizations and functions of alternative gene products, which arise from alterations in the gene transcript (see 2.3.1). The underlying mechanisms in this latter case may however still be based on either a mechanism requiring a "second hit" or haploinsufficiency of the gene product.

2.3   Neurofibromin and the GTPase activating domain

The protein encoded by the *NF1* gene, neurofibromin, consists of 2,818 aminoacids with a predicted molecular mass of 327 kDa (136). A 360 amino acid region of the predicted protein

product shows homology to the GTPase activating (GAP) family of proteins in yeast and mammals (137). The GAP-related domain (*NF1*-GRD) of neurofibromin is the only known functional domain of the *NF1* gene and spans exons 20 to 27a (bases 3497 - 4661) (figure 1.2.1) (138).

The *NF1*-GRD of neurofibromin interacts with the product of the *RAS* protooncogene by binding to RAS and accelerating the hydrolysis of active RAS-Guanosine Triphosphate (RAS-GTP) into inactive RAS-Guanosine Diphosphate (RAS-GDP) (figure 1.2.4). By cycling between an active and an inactive form, the *RAS* family of proto-oncogenes are regulators of cellular proliferation and differentiation. Somatic mutations, causing loss or mutation of the wild type allele may lead to loss of function of neurofibromin. Studies in neurofibrosarcoma and leukemic cells have shown that loss of neurofibromin leads to high levels of active RAS-GTP and increases in cellular proliferation (139, 140), synonymous with a loss of tumor suppression. Contrastingly, other studies in neuroblastoma and melanoma cell lines show loss of neurofibromin but normal levels of RAS-GTP (141). Hypotheses are that neurofibromin regulates growth by mechanisms that are both RAS-GTP dependent (neurofibrosarcoma and leukemic cell lines) and RAS-GTP independent (neuroblastoma and melanoma cell lines).
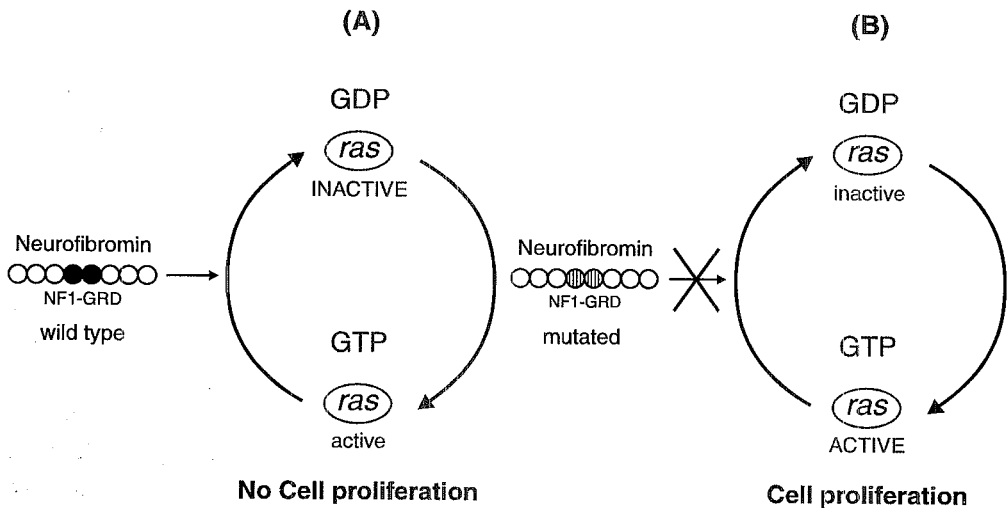

Figure 1.2.4
Figure 1.2.4 A) Hydrolysis of active RAS-GTP into inactive RAS-GDP by neurofibromin. Cell proliferation is regulated. Figure 1.2.4 B) Loss of function of neurofibromin leads to high levels of active RAS-GTP. Cell proliferation is no longer regulated.

Classical differential centrifugation and glycerol gradients showed a cytoplasmic localization for neurofibromin (142), and indirect immunofluorescent analyses using antibodies against neuro-fibromin demonstrated its association with cytoplasmic microtubules (143). The residues responsible for microtubule interaction reside within the *NF1*-GRD of the gene product. This association suggests that neurofibromin is involved in microtubule-mediated intracellular signal transduction pathways (143).

2.3.1 Alternative gene products

Alternative *NF1* gene products have been reported in specific tissues at different developmental stages. Although little is known of the pathogenesis of NF1, it is probable that mechanisms leading to illegitimate expression or lack of alternative gene transcripts at different stages of development may play a role in the clinical variability of the disease. For example, an alternative gene product lacking a *NF1*-GRD region in a tissue at a period during development that *NF1*-GRD is obligatory, may cause clinical manifestations. It is well known that a single gene possesses methods to create varying proteins. Widely studied mechanisms are alternative splicing of pre-mRNA's and alternative polyadenylation site selection. Recently, another pathway leading to RNA sequence diversity was discovered: mRNA editing.

Four alternative splice forms of the *NF1* gene transcript have been reported, leading to five different neurofibromin gene products; the most common product being GRD1 (table 1.2.2) (137). One study showed that the expression of the gene products GRD1 and GRD2 was associated with the differentiation stage of the tissue. Predominant expression of GRD1 was reported in fetal brain and undifferentiated primitive neuroectodermal tumors. Contrastingly, GRD2 was predominantly observed in adult brain and differentiated cell lines (144). However, Suzuki et al. (145) reported higher GRD1/GRD2 ratios in adult brain and preferential expression of GRD2 in primary brain tumors. The third alternative gene product, 3'ALT, showed its highest expression in adult cardiac muscle, skeletal muscle and smooth muscle of the bladder with low levels in brain and nerve (146). Replacement of exon 11 results in a truncated transcript of 2.9 kb and a fourth alternative protein product (5'ALT) which is devoid of a major C-terminal part of neurofibromin including the *NF1*-GRD; no tissue localizations have been reported for 5'ALT (147). Lastly, a fifth *NF1* gene product (5'ALT2) seems solely expressed in the central nervous system. Expression is decreased in undifferentiated tumors such as medulloblastoma and oligo-dendrogliomas (148).

Table 1.2.2
Sequence alterations and expression patterns
of alternative neurofibromin splice forms

Abbreviation	Sequence alteration	Expression in:
GRD1	most common gene product	fetal and adult brain, undifferentiated primitive neuroectodermal tumors
GRD2	additional exon 23a (+ 63 bp)	adult brain, differentiated cell lines, primary brain tumors
3'ALT	additional exon 48a (+ 54 bp)	highest in muscles (cardiac, skeletal and smooth muscles)
5'ALT1	replacement exon 11 (truncated transcript of 2.9 kb) no *NF1*-GRD	
5'ALT2	additional exon 9br (+ 30 bp)	central nervous system (lower levels in medulloblastoma and oligodendrogliomas)

bp = base pairs; kb = kilobase

A second mechanism which leads to alternative gene products at RNA level is alternative polyadenylation site selection. At a polyadenylation site in the primary transcript, the mRNA may be terminated and given a poly A tail. The choice of a polyadenylation site is regulated; alternative polyadenylation sites will produce proteins of different lengths and functions (149).

A third and recently described mechanism is mRNA editing. In mRNA editing, posttranscriptional modifications are made of the gene transcript. During translation by the ribosomes, alterations in the mRNA sequence are transferred into the gene product (150). In the *NF1* gene, an mRNA editing site was discovered within the *NF1* mRNA at the N-terminal region of the *NF1*-GRD (exon 23) (151). Editing at this site would lead to an in-frame translation stop codon and would produce a truncated protein. Truncation of the transcript derived from the wild type allele (in the *NF1*-GRD), would result in complete inactivation of the tumor suppressor function of the *NF1* gene in that cell or tissue. Therefore, mRNA editing may lead to a functional equivalent of biallelic inactivation of the *NF1* gene. Studies of NF1-related tumors showed highest levels of mRNA editing in malignant tumors, lowest in dermal neurofibromas and intermediate levels in plexiform neurofibromas (151). These results suggest an important role for mRNA editing in tumor formation.

In conclusion, a number of alternative gene products are known. However, although different expression patterns have been found for some alternative gene products, their functions and effects on the phenotype are still to be elucidated.

2.4   Animal models

Availability of animal models may be of benefit for studying NF1 for the following reasons. The natural history of the disease might be followed if an *NF1* gene mutation is adequately expressed. More importantly, factors contributing to tumor formation and growth may be studied. As mutations may be induced, relationships between specific types of mutations, the resulting protein and clinical severity can be analyzed (genotype-phenotype correlations) as well as possible influences of other (modifying) genes. Also, early postembryonic and fetal development in animals with NF1 may be studied. In the future, the effects of treatment and environmental influences can be observed *in vivo* and *in vitro*.

There are several naturally occurring animal models with NF1 (152). Neurofibromas have been reported in birds, various mammals and fish species, but are generally non-inheritable. A genetic form of NF1 has been observed in Holstein cows, presenting as dermal and plexiform neurofibromas, malignant peripheral nerve sheath tumors and possibly bovine Lisch nodules (153). Of special interest is also the bicolor damselfish that may present with pigmentary changes, neurofibromas, schwannomas and malignant peripheral nerve sheath tumors (154). However, epizootic and tumor transplant data have suggested that the symptoms in bicolor damselfish have an infectious etiology (155).

Four types of mouse models have been established: NF1 -/- embryos, NF1 +/- animals, NF1 -/- chimeras and mice transplanted with NF1 -/- hematopoetic stem cells. Expectations of mouse models were high, as there is a high level of conservation between mouse and human neurofibromin (98% homology) (156).

Brannan et al. (157) developed a mouse homozygous for an *NF1* gene mutation by targeted disruption of the murine *NF1* gene in embryonic stem (ES) cells. These neurofibromin deficient mice all died in the embryonic stage due to widespread developmental abnormalities. Detailed analysis revealed normal development until day 12 of embryogenesis when the mice became pale and edematous. Histological studies showed major defects of the heart and large vessels leading to pericardial effusion, cardiac failure and death at day 13.5. In

addition, a hyperplasia was observed of neural crest derived sympathetic ganglia and developmental delay of a number of organ systems (renal, hepatic and skeletal muscle systems). Another study could not confirm these latter observations (158). Human NF1 is caused by a heterozygous mutational status and cardiac abnormalities are uncommon. Still, the homozygous knock-out mouse underlines the importance of functional neural crest derivatives in the formation of the outflow tracts of the heart (also see table 1.2.1).

Mice heterozygous for a *NF1* gene mutation may be more suitable models for human NF1. Jacks et al. (158) disrupted the murine nf1 locus in the equivalent of exon 31 in ES cells, the site of several *NF1* gene mutations in humans. More than 250 heterozygous mice followed-up for a long period of time (7 months to 2 years) showed no hyperpigmentation, neurofibromas or Lisch nodules. However, 75% showed tumorigenesis versus 15% in controls, supporting the putative role of the *NF1* gene as a tumor suppressor gene. The spectrum of neoplasms was similar to that observed in older normal controls, suggesting an acceleration in the development of tumor types for which mice have a natural susceptibility. Higher incidences than in controls were reported for pheochromocytoma and myeloid leukaemia. These 2 tumor types are specifically seen at an increased rate in human NF1. Recently, the NF1 mice were shown to have similar learning disabilities as in human NF1. The learning and memory deficits were limited to specific functions and could be compensated with remedial training of these functions (159).

Recently, NF1 -/- chimeric mice were generated, some of which survived until birth and some until adulthood (T. Jacks, personal communication).

In summary, homozygous NF1 mice models show embryonic lethality, that is not known in human NF1 probably because of the very rare NF1 x NF1 matings. Although heterozygous models lack the typical skin manifestations, probably through differences in regulatory systems for cell growth and differentiation in mice, they do show similarities to human NF1 in tumorigenesis and learning difficulties. Therefore, the heterozygous mouse model may allow analysis of the mechanisms concerning these NF1-related symptoms and testing of therapeutic approaches.

2.5 Mutation detection

As mutations in the *NF1* gene vary from large to small deletions, insertions, point mutations and a few mutational "hot spots", detection methods are necessarily diverse. Large deletions have been detected by analysis with pulsed field gel electrophoresis (PFGE), intragenic polymorphic markers, by Southern blotting using restriction enzymes and intragenic cDNA probes. Recently, fluorescence *in situ* hybridization (FISH) with cosmid-and yeast artificial chromosome (YAC) clones was used for deletion detection. Smaller mutations, which seem to form the majority of mutations (table 1.2.3) are detected by single strand conformational analysis (SSCP) (160), heteroduplex analysis (HA) (104), denaturing gradient gel electrophoresis (DGGE) (102), temperature gradient gel electrophoresis (TGGE) (105) and chemical mismatch cleavage (CMC) (161). SSCP and HA are by far the most applied techniques.

Table 1.2.3
Mutation types reported to the
NF1 Genetic Analysis Consortium by April 1997 (108)

Mutation type	n
Chromosomal rearrangement involving *NF1* gene	4
Deletion entire gene	23
Small deletion	66
Large insertion	3
Small insertion	25
Nonsense mutation	38
Amino acid substitution	23
Intron mutation	23
3"UTR mutation	4
Total	**229**

The protein truncation test (PTT), a technique based on a combination of RT-PCR of RNA (in 5 overlapping segments) with *in vitro* transcription and translation, detects mutations causing a premature stop codon and thus a truncated protein. One study reported that 67% of mutations could be detected in a series of NF1-patients (14/21) using the PTT (162). It was concluded that the majority of mutations were nonsense mutations. The lack of further mutation reports using this technique suggests that it is less suitable for screening blood samples of large groups of NF1-patients. More recently, a technique was developed based on amplification of the entire protein coding sequence as a single PCR product by RT-PCR.

Digestion with restriction enzymes and electrophoresis on agarose shows aberrant bands in the presence of insertion/ deletion mutations in the mRNA. It was concluded that this technique may detect 30% of all previously reported mutations (163).

Despite the development of new techniques, detection rates of mutations in the *NF1* gene in clinical series of NF1-patients are still between 10-20% (164). The most important reasons for this low detection rate are the large size of the gene, the high frequency of small mutations and the paucity of recurrent mutations. Screening of 60 exons is a time consuming, laborious and expensive task and is seldomly accomplished. In practice, mutation analysis consists of random screening of exons of large groups of patients. Most studied exons are situated in or in the vicinity of the *NF1*-GRD. It may be possible that mutational "hot spots" will be discovered in less intensively screened exons in the near future.

The NF1 Genetic Analysis Consortium, in which the Department of Clinical Genetics of the Erasmus University participates, was founded in order to share information on detected mutations, polymorphisms and techniques. In April 1997, 229 mostly different mutations had been reported to the Consortium (108). A classification of mutation types is given in table 1.2.3 The Consortium is sponsored by National Neurofibromatosis Foundation Inc.

2.6   Genotype-phenotype correlations

On the basis of the extreme clinical variability within NF1 families, it was expected that genotype-phenotype studies would be complex and not straightforward. In addition, the high percentage of de novo cases, the large size of the *NF1* gene, the paucity of large NF1 kindreds, and the diversity of mutations impedes the frequent detection of (identical) mutations in related- and unrelated individuals, making large genotype-phenotype studies difficult. Similar observations have been reported for other autosomal dominant diseases such as tuberous sclerosis (165) and the Marfan syndrome (166).

Genotype-phenotype correlations in NF1 seem most feasible in those patients in whom deletions of the *NF1*-GRD, of the embedded genes *EVI2A* and *EVI2B* (human homologues of murine genes involved in leukemia) and *OMGP* (encodes a glycoprotein involved in myelinisation), deletions of the entire *NF1* gene, or identical (recurrent) or similar mutations (affecting the same codon) were detected.

However, even though direct relationships may not be found, genotype and phenotype studies remain important. Continuation of these studies is indispensable for a further understanding of the pathogenesis of NF1 and may uncover interactions and possible modifiers, providing new insights in the clinical expression of the disease.

2.6.1   Modifying genes

To investigate if the phenotypic variation of NF1 has a genetic component, Easton et al. scored quantitive traits (number of *café-au-lait* spots, number of dermal neurofibromas, head circumference) and binary traits (presence or absence of plexiform neurofibromas, optic gliomas, scoliosis, epilepsy and referral for remedial education) in 175 individuals from 48 NF1 families. The phenotypic correlation decreased with the degree of relationship, being strongest between monozygous twins and zero between distant relatives. Therefore, the variation in phenotype is likely to be influenced by genetic factors unlinked to the *NF1* locus (167).

Possible candidates could be factors involved at any level of the correct generation of (tissue specific) gene products varying from transcription to posttranscriptional and posttranslational modification (see 2.3.1). These modulators could affect alternative splicing, polyadenylation site selection and mRNA editing. Phosphorylation sites located on neurofibromin also suggest possible regulation by kinases (168).

Furthermore, studies have shown that certain growth factors such as platelet derived growth factor (PDGF) and transforming growth factor beta 1 may be implicated in the development of neurofibromas (169). cDNA of stem cell factor, the receptor which is encoded by the c-kit gene, was expressed more strongly in neurofibroma tissue than in normal tissue (170). The development of mast cells, a major component of neurofibromas, is induced by this stem cell factor. Also, *NF1* gene loss may induce JCML through a hypersensitivity to granulocyte/ macrophage-colony stimulating factor (GM-CSF) (171).

As neurofibromin is involved in microtubule-mediated intracellular signal transduction pathways (143), major candidates formodifying genes(see 2.6.1) may be sought among the genes encoding proteins interacting with neurofibromin in signal transduction. However, little is known of these pathways to date (172). More research is clearly necessary to unravel the complex interactions between the products of the *NF1* gene (*NF1*-GRD and other yet unidentified domains) and other gene products.

3.1   Clinical setting

Since 1985, a multidisciplinary Neurofibromatosis (NF) team (coordinated by a pediatrician, and including a dermatologist, pediatric neurologist, ophthalmologist, clinical geneticist and molecular geneticist) in Sophia Children's Hospital has been active in supraregional diagnostic referral and evaluation of children suspected of or affected with NF1.

Prior to January 1st 1996, 209 children were examined for a suspected diagnosis of NF1. In 150 children (64 girls, 86 boys) NF1 was diagnosed according to NIH criteria; 59 children did not meet diagnostic criteria for NF1. Almost half of the NF1-affected children were referred by a general practitioner; the other half by medical specialists. At the first visit, clinical examinations by the various specialists, X-rays of the skull and the entire spine and visual evoked potential (VEP) were performed. In all children, irrespective of the presence of complications, follow-up consisted of periodic outpatient clinical evaluations supplemented by additional studies as required. This setting made a 10 year prospective follow-up study possible and has led to the largest single center study of NF1-affected children followed until 18 years of age.

3.2 *NF1* linkage- and mutation analysis

In this study, linkage- and mutation analysis was performed on the DNA of NF1-patients and their families, referred by specialists (including clinical geneticists) from throughout the Netherlands. Many of the probands were recruited by the multidisciplinary NF team in the Sophia Children's Hospital and the Clinical Genetics Department in Rotterdam. Prior to April 1, 1997, DNA samples of 200 unrelated NF1-patients and their families had been collected for analysis. Linkage analysis was primarily performed in large kindreds with intragenic and (when necessary) extragenic markers. The feasability of linkage analysis is dependent on the family structure and informativeness of a series of intragenic markers (chapters 7 and 8). DNA of NF1-patients was subsequently screened for large and small mutations. Large mutations were detected by intragenic markers and Southern blotting techniques. Smaller mutations were detected by SSCP analysis, analyzed by sequencing techniques and confirmed by allele specific oligonucleotide analysis (ASO).

3.3 Outline and aims

The **clinical** research objectives included:
1. Analysis of the prevalence and incidence of symptoms and specific complications in children with NF1.
2. Evaluation of possible risk factors for the development of complications, eventually enabling early recognition and possible intervention.
3. Recognition of specific phenotypes to further analyze clinical heterogeneity.
4. Evaluation of a multidisciplinary NF1 clinic in the early diagnosis of NF1 and information of parents, relatives, general practitioners and medical specialists.

The **molecular genetic** objectives included:
1.   linkage- and mutation analysis for diagnosis, including presymptomatic and prenatal diagnosis.
2.   Genotype-phenotype studies.

Outline of results and discussion of the study
Results of **10 years follow-up of 150 children with NF1** by a multidisciplinary NF team are reported in **chapter 2**. This study allowed transversal and longitudinal analysis of complications related to NF1 and possible risk factors involved. Also, the value of a **comprehensive diagnostic- and follow-up clinic** is discussed. It is the first prospective study on the actual occurrence of symptoms and complications in such a large group of children during a 10 year period. The **value of the minor disease features**: macrocephaly, short stature, hypertelorism and thorax abnormalities **in predicting a diagnosis of NF1** in children $\leq$ 6 years suspected of NF1 is discussed in **chapter 3**.

Selected NF1- specific complications were adressed in two studies. **Endocrine problems** like precocious puberty are established NF1 complications, clinically associated with optic pathway glioma. In this study however, **central precocious puberty** (CPP) was observed in the absence of an optic pathway glioma. In addition, the **first prevalence figures** of **growth hormone deficiency** (GHD) (not secondary to radiotherapy) could be established in this large series of NF1-affected children (**chapter 4**).

Atlantoaxial dislocation is related to cervical dysplasias, and can be a dangerous complication for instance during surgery for a plexiform neurofibroma in the neck region. The frequency of cervical vertebral anomalies in NF1 (10%) illustrates their importance in the perioperative management of NF1-patients (**chapter 5**).

The variety of slowly developing symptoms during childhood is one factor causing **diagnostic delay** observed in referred pediatric NF1-patients and their families. Half of the pediatric and one third of the adult patients were treated for symptoms related to NF1 before a specific diagnosis was made (**chapter 6**).

The **molecular genetic studies in 200 patients** and families (**chapters 7 and 8**) allowed a genotype-phenotype study of 8 unrelated patients with **deletions encompassing the entire gene**. Strikingly, 7/8 showed a phenotype of intellectual impairment and dysmorphic features, suggestive for a severe expression of the disorder, as was also found in other studies. In addition, the **recurrent mutation R1947X** was detected in 4 probands. The variable phenotype in these patients was compared to other reports. Moreover, **6 novel mutations**

and 1 possible mutation are reported. This series contributes 10% of the mutations known in the NF1 Genetic Analysis Consortium registry (**chapter 8**).

The general discussion reflects on the clinical, diagnostic and genetic problems of NF1. The most important effects of molecular genetic understanding are still to be awaited, as the knowledge on this frequent, often severe genetic disorder is still very incomplete (**chapter 9**).

The thesis concludes with **summaries** in English and Dutch (**chapter 10**).

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Chapter 1

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Chapter 2

A prospective 10 year follow-up study
of patients with Neurofibromatosis type 1

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Abstract

Objective To establish the prevalence and incidence of symptoms and complications in children with Neurofibromatosis type 1 (NF1) and to assess possible risk factors for the development of complications.

Design A 10 year prospective multidisciplinary follow-up study.

Patients 150 children diagnosed with NF1 according to criteria set by the National Institutes of Health.

Results In 62/150 children (41.3%) 1 or more complications were present, including 42 (28.0%) children with 1 complication, 18 (12.0%) with 2 complications and 2 (1.3%) children with 3 complications (mean duration of follow-up: 4.9 years ;standard deviation = 3.8). Ninety five of the 150 children presented without complications (follow-up: 340.8 person years). Incidence of complications was 2.4 complication per 100 person years in this group. An association was found between behavioral problems and presence of complications.

Conclusion This is the largest single center case-series of NF1-affected children followed until 18 years of age. Based on our data, we recommend regular clinical examinations in children with NF1, also in children initially not presenting with complications.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder, affecting 1:3300 individuals. Half of all cases represent sporadic cases (1). Symptoms are highly variable and severity cannot be predicted, even within families. Diagnostic criteria for NF1 have been established by the National Institutes of Health Consensus Development Conference (NIH) in 1988 (2).

Characteristic features of the disease include *café-au-lait* spots, axillary and inguinal freckling, neurofibromas and Lisch nodules. Minor disease features observed in NF1-patients are macrocephaly, short stature, hypertelorism, thorax abnormalities, learning-, speech-, motor-, behavioral- and disorders. In childhood, various complications may occur such as mental retardation, tumors of the central nervous system (optic and chiasm gliomas), orthopaedic abnormalities, endocrinological disorders and malignancies (3, 4).

Previously, only prevalences of symptoms and complications were reported in NF1-patients. In this study, which includes the largest reported series of children with NF1, we determined both overall prevalence and incidence rates of symptoms and complications in 150 children with NF1. Subsequently, an analysis was conducted of possible risk factors for the development of complications in children with NF1.

Patients and Methods

Patients and follow-up

Since 1985, a multidisciplinary NF1-team in the Sophia Children's University Hospital in Rotterdam including a pediatrician, dermatologist, pediatric neurologist, ophthalmologist and clinical geneticist, has evaluated children suspected of NF1. At first visit clinical evaluations, X-rays of the cranium and the entire spine and a visual evoked potential (VEP) were performed. In all NF1-affected children, irrespective of presence of complications, follow-up consisted of periodic clinical evaluations supplemented by additional studies as required. In this study, follow-up was calculated from first examination date with an NF1 diagnosis.

CT- and MRI-scanning of the brain was only performed upon indication. Indications included ophthalmological abnormalities (optic atrophy, relative afferent pupillary defect, abnormal Ishihara color vision test or abnormal VEP), endocrinological disorders, mental retardation, and various other neurological abnormalities.

Definitions

Symptoms and complications observed in this study included various learning problems, speech-, motor- and behavioral disorders, mental retardation, optic pathway glioma, epilepsy, plexiform neurofibroma, bowing of tibia with or without pseudoarthrosis, hemihypertrophy, severe scoliosis, aqueduct stenosis, atlantoaxial dislocation, endocrinological disorders and malignancies.

Individual symptoms and complications were defined as follows.
Mental retardation was defined as attendance of schooling for the mentally disabled, learning difficulties as attendance of special schooling, other than schooling for the mentally disabled. Accordingly, prevalences of mental retardation and learning difficulties were established in children 6 years and older (n = 110). Speech abnormalities were variable and often included late onset of speech as well as articulatory problems. Counseling by a speech therapist was recorded.

Motor problems were defined as clumsiness on evaluation by a pediatric neurologist, support by a physical therapist was reported. Behavioral problems differed from attention-deficit-hyperactivity-disorders (ADHD) to aggressive behavior and depressive feelings requiring psychiatric help.

Optic pathway glioma reported in this study, were detected on CT/MRI scans of the brain.

Plexiform neurofibroma were defined as either a large, soft swelling with ill defined borders (diffuse plexiform neurofibroma) or a firm, spherical or ovoid shaped swelling (nodular plexiform neurofibroma) (4). Plexiform neurofibromas were interpreted as complications on the basis of either localization or symptomatology. Threatening localizations included the head and neck, in the vertebral column, paravertebrally and/ or in the perineal region. Plexiform neurofibromas causing severe symptoms (pain, functional disorders, neurological deficits and cosmetic disfigurement) were also considered complications.

Severe scoliosis was defined as a vertebral column abnormality requiring therapy, such as surgery or corset.

Prevalence rates

Prevalence was defined as the existence of a symptom or complication on January 1st 1996 in *children diagnosed with NF1 according to NIH criteria and younger than 18 years of age*. Nine children, examined before 1985 by all specialists on a regular basis, were also included in this study. This population will be referred to as **group A** (n = 150).

The prevalence of symptoms and complications at initial presentation can easily be calculated by subtracting the number of occurring symptoms/ complications (n) from the overall prevalence rate.

Incidence rates

Incidence rates of occurring symptoms and complications were established on January 1st 1996 in *children without complications at presentation, diagnosed with NF1 and younger than 18 years of age*. This group of children, which is a subset of group A, will be called **group B** (n = 95). These patients presented solely with *café-au-lait* spots, freckling, Lisch nodules and/or dermal neurofibromas. Person years, the denominator of the incidence rate, was defined by the first examination date with NF1 diagnosis and last examination date. Children reaching the age of 18 years before January 1996 were considered withdrawn alive.

Incidence rates of newly presenting complications were also computed in children with 1 or 2 complications at presentation. This group will be referred to as **group C** (n = 55) and is also a subset of group A. Person years were defined by first examination with NF1 diagnosis and complication(s) and last examination date. Complications were defined as new when occurring more than 1 year after the presenting complication(s).

Analysis

Clinical data were obtained from medical records and registered on a standardized form. Data were analyzed with Dbase IV and SPSS 6.0. Associations between specific symptoms and the presence of complications were assessed by univariate analysis with the chi-square test (p-value = 0.05, two-sided). Risk factors tested included sex, age at NIH diagnosis, physical characteristics observed in children with NF1, such as macrocephaly, short stature, hypertelorism, thorax abnormalities and mild scoliosis as well as a variety of symptoms such as learning difficulties, motor disorders, behavioral problems and speech disorders.

Results

During this prospective follow-up study 209 children were examined for a suspected diagnosis of NF1. In 150 children (64 girls, 86 boys) NF1 was diagnosed according to NIH criteria (table 2.1). In 59 children the diagnosis could not be made. Five children (3.3%) died of malignancies during the follow-up period. Almost half of the NF1-affected children (46%) were referred by either a general practitioner or other primary health care workers. The other half were referred by medical specialists.

Prevalence rates

Prevalence rates of diagnostic criteria and complications in group A are depicted in table 2.2 Mean age at last examination date was 10.4 years (standard deviation (SD) 5.1). Mean duration of follow-up was 4.9 years (SD 3.8).

Diagnostic criteria

Six or more *café-au-lait* spots of the required diameter were diagnosed in 96.7% of NF1-affected children (mean age at presentation of sign: 3.1 years; SD = 3.1). Freckling was diagnosed in 85.3% (mean age at presentation of sign: 6.3 years; SD 4.2), dermal neurofibromas in 40.0% (mean age at presentation of sign: 8.9 years: SD 4.5), plexiform neurofibromas in 26.6%, Lisch

Table 2.1
NIH Diagnostic criteria in NF1

2 or more of the following criteria are necessary for a diagnosis of NF1

Clinical signs

-   6 or more café-au-lait spots over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals.

-   Two or more neurofibromas of any type or one or more plexiform neurofibromas.

-   Freckling in the axillary or inguinal region.

-   Optic or chiasma glioma.

-   Two or more Lisch nodules (iris hamartomas).

-   A distinctive osseous lesion, such as sphenoid dysplasia or thinning of long bone cortex, with or without bowing and pseudoarthrosis.

Family History

-   A first-degree relative (parent, sibling or offspring) with NF1 by the above criteria.

nodules in 52.0% (mean age at presentation of sign: 8.8; SD 3.6) and a distinctive osseous lesion (sphenoid dysplasia, thinning of long bone cortex) was observed in 7.8% of NF1-affected children (table 2.2). In addition, scalloping of the vertebral bodies was seen in 5 (4.0%) children and widened foramina of the vertebral bodies in 10 (7.8%) children. In 74/150 (49.3%) cases one or more family members were also diagnosed with NF1.

Minor disease features
Learning difficulties, speech abnormalities, behavioral problems and motor disorders were observed in respectively, 33.0%, 58.7%, 47.3% and 49.3%. In 76% of the children with speech abnormalities a speech therapist was visited. Support by a physical therapist was recorded in 66% of the children with motor disorders. Data on macrocephaly, short stature, hypertelorism and thorax abnormalities have been reported elsewhere (5).

Complications
In summary, in group A, 62/150 (41.3%) showed 1 or more complications, including 42 (28.0%) children with 1 complication, 18 (12.0%) with 2 complications and 2 (1.3%) children with 3 complications. In 88/150 (58.7%) no complications had been diagnosed at last examination date.

Table 2.2
Prevalence rates of diagnostic criteria, symptoms
and complications in 150 unselected NF1-children (Group A)

	% (n)	Huson et al. [5] %	Obringer et al. %
Diagnostic criteria			
Café-au-lait spots	96.7 (145)	97-100	97.0
Freckling	85.3 (128)	70.0	81.0
Neurofibromas			
- dermal	40.0 (60)	0-40	15.0
- plexiform [1]	26.6 (40)	25.7	..
Lisch nodules	52.0 (78)	79-85	30.0
Distinctive osseous lesion [2]	7.8 (11)	-	6.0
Complications			
Mental retardation	17.2 (19)	2.9	..
Optic pathway glioma	11.3 (17)	5.1	4.0
Epilepsy	0.7 (1)	5.1	-
Plexiform neurofibroma [3]	18.0 (27)	12.8	-
Bowing	2.7 (4)	-	
Pseudoarthrosis	2.0 (3)	7.8	6.0
Hemihypertrophy	2.7 (4)	-	..
Severe scoliosis	2.0 (3)	2.6	..
Aqueduct stenosis	0.7 (1)	2.1	..
Atlantoaxial dislocation	0.7 (1)	-	..
Endocrinological disorder	4.6 (7)	-	-
Central precocious puberty	2.0 (3)	-	-
Growth hormone deficiency	2.0 (3)	-	..
Diencephalic syndrome	0.7 (1)	-	-
Malignancy [4]	4.0 (6)	1.5 [6]	5.0
Neurofibrosarcoma	2.0 (3)	-	-
Malignant brain tumour	2.7 (4)	-	..

[1]   Total number of plexiform neurofibroma.
[2]   Sphenoid and occipital dysplasia (number of X-rays/ CT-scans skull = 138).
[3]   Plexiform neurofibromas considered as complications.
[4]   One child was diagnosed with both an astrocytoma and a neurofibrosarcoma.
[5]   If available, percentages shown in NF1-patients ≤ 18 years (n = 39).
[6]   Both rhabdomyosarcoma.

Ophthalmological symptoms were observed in 10 of the 17 children with an optic pathway glioma (58.8%). In total, 3 of the 17 (17.6%) children with an optic pathway glioma received radiotherapy due to progressive visual loss. All but one child with an optic pathway glioma had abnormal visual evoked potential (VEP) at first presentation. Children with an endocrinological disorder after radiotherapy for an optic pathway glioma were not included in this analysis (n = 2).

In 3 of 4 children (75.0%) with a bowing of the tibia, a pseudoarthrosis developed. One child presented with bilateral bowing and unilateral pseudoarthrosis, which is a rare condition. Histology reports of malignant brain tumors included 2 astrocytomas, 1 medulloblastoma, 1 oligodendroglioma (grade III).

Incidence rates
Symptoms and complications
The total number of person years in children presenting without complications was 340.8 person years. The incidence of complications in group B is depicted in table 2.3. In group B, 2.4 complication developed per 100 person years. Complications developed in 7 children; in 6 children 1 complication developed, in one child 2 complications developed. Incidence rates were highest for optic pathway glioma and plexiform neurofibroma. Univariate analysis for possible risk factors in group B, showed an association between behavioral problems and the presence of complications (chi-square test; p-value < 0.05).

Subsequent complications in children with 1 or 2 complications at presentation are depicted in table 2.4. The total number of person years in this subset was 322.6 person years. In group C, 3.0 complication developed per 100 person years. The majority of patients developed an endocrinological disorder or a malignancy as second/ third complication. One patient with cervical dysplasias at presentation experienced an atlantoaxial dislocation after surgery for a plexiform neurofibroma (6). There was no significant difference between the number of complications which developed in group A and group B (95% CI: -0.05; 0.07; p-value > 0.05).

Table 2.3

Incidence rates of complications in 95 children presenting without complications (Group B)

	Incidence rates/ per 100 person years (n)
Optic pathway glioma	1.2 (4)
Plexiform neurofibroma	0.6 (2)
Atlantoaxial dislocation	0.3 (1)
Endocrinological disorder	0.3 (1)

Table 2.4

Incidence rates of subsequent complications
in 55 children presenting with 1 or 2 complications (Group C)

	Incidence rate/ per 100 person years (n)
Optic pathway glioma	0.3 (1)
Plexiform neurofibroma	0.3 (1)
Severe scoliosis	0.3 (1)
Atlantoaxial dislocation	0.3 (1)
Endocrinological disorder	0.9 (3)
Malignancy	0.9 (3)

Discussion

This study presents prevalences as well as incidence rates of symptoms and complications in children with NF1, recorded during a prospective follow-up study of a large group of NF1-affected children (n = 150). Huson et al. (7) documented the age range of presentation and

prevalence of major complications in NF1 in a smaller group of 39 NF1-patients younger than 18 years. Long term prospective follow-up of children with NF1 was determined necessary to define the natural history of the disease. An analysis of the prevalence and applicability of the

diagnostic criteria in children of 6 years old and younger was given by Obringer et al. (8). Descriptions of complications were however not included.

Prevalence rates of symptoms and complications
Strikingly, in the study population as a whole, complications were observed in 42%; moreover, one third of these children had 2 or more complications of the disease.

The prevalences of the diagnostic criteria and severe scoliosis were similar to those reported in other studies (2, 7, 8).

Prevalence rates of learning difficulties, motor disorders, behavioral problems and speech disorders were similar to those reported in other studies (9, 10). The high percentages of these problems emphasize the necessity of follow-up of NF1-affected children and extensive information of parents on the association of these symptoms with the disease so that educational and supportive measures can be taken.

The prevalence of optic pathway glioma is variously reported, depending on the indication for brain imaging in NF1: if done routinely 19% (11), if done because of suspected central nervous system tumors, eye problems and/ or endocrinological abnormalities 11.3% (this study) or because of suspected eye problems 5.1% (7). We agree that routine neuroimaging for optic pathway glioma is not indicated as only half of the detected optic pathway glioma in our study were symptomatic; one third of these presented with progressive visual loss. In only 1 of the 3 children in this series, with central precocious puberty an optic pathway glioma was found on MRI-examination. This is in contrast with earlier observations of concurrence of chiasma glioma in all NF1-patients with central precocious puberty (12).

Distinct from Huson et al. (7), none of the NF1-patients in this series showed rhabdomyosarcoma. Therefore, our data suggest that this is a rare complication in children with NF1. Prevalences of both epilepsy, pseudoarthrosis and aqueduct stenosis were higher in the Welsh study. Contrastingly, except for 2 patients with delayed puberty, Huson did not observe any children with endocrinological disorders (7).

Incidence rates of symptoms and complications
In the group presenting without complications, 2.4 complication developed per 100 person years. By way of illustration, follow-up of 20 children, presenting without complications for 5 years would lead to development of more than 2 complications in the total group. This may not seem

impressive but the complications in NF1 are severe and cause both serious morbidity and mortality. We feel follow-up of children is indicated on the basis of these data.

The incidence rate of subsequent complications in children presenting with 1 or 2 complications was 3.0 complication per 100 person years and was not significantly higher than in the group presenting without complications.

As this is the first study on incidence rates of symptoms and complications in NF1-patients, there are no literature data available. Although the majority of plexiform neurofibromas (88.9%) and optic pathway gliomas (70.6%) presented with symptoms at initial examination, they may also develop symptoms later on and present during follow-up. Accordingly, optic pathway gliomas have also been reported to develop in patients without abnormalities on MRI scan at presentation (13).

The majority of endocrinological disorders (57.1%) however, presented during follow-up (also see table 2.4). Deviations from the growth velocity curve and premature manifestations of puberty should therefore be recognized as possible complications of NF1 on the basis of an endocrinological disorder. Malignancies were all acquired during follow-up. Half of presenting tumors developed in preexisting plexiform neurofibromas.

An association was found between behavioral problems and the development of complications. However, behavioral problems may also be a consequence of complications in children and as such cannot be considered a risk factor.

Future prospects and recommendations
The data in this study indicate that routine X-rays of the cranium and the entire spine are unnecessary, unless of course symptoms are present giving an indication for these investigations. The observation of one case of atlantoaxial dislocation in a patient with NF1 and cervical dysplasias and the 11.8% prevalence of this symptom in NF1 leads to recommendation of complete bidirectional cervical vertebral column X-rays in all NF1-patients undergoing anaesthesia and/ or surgery. Visual evoked potentials were a valuable addition to the ophthalmological examination at first presentation. Although the specificity of this test is low, sensitivity is high (14), leading to detection and treatment of progressive optic pathway glioma in an early stage.

The present data give the most comprehensive justification for follow-up management of NF1-patients as it gives the prevalence of complications at presentation, the increase of complications (incidence rate) and the overall prevalence of specific complications. We conclude that NF1 is

a disease that is associated with various psychomotor problems and severe complications during childhood. Both merit regular screening by an experienced pediatrician, pediatric neurologist and ophthalmologist, also in children initially not presenting with complications. Factors predisposing children with NF1 towards the development of complications remain to be determined. Children without complications may be seen every 1 to 2 years, unless problems arise earlier. Children with complications at presentation should be examined more frequently. These children do not have a higher risk of subsequent complications than children presenting without complications. We hope that our prospective follow-up study will be useful for physicians and clinical geneticists caring for NF1-patients and informing families on the disease and it's complications.

Acknowledgements

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Chapter 3

Minor disease features
in Neurofibromatosis type 1 (NF1)
and their possible value in diagnosis of NF1
in children ≤ 6 years and clinically suspected of NF1

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Abstract

Objective To establish the frequency of minor disease features in children with Neurofibromatosis type 1 (NF1) and to evaluate the value of minor disease features in children ≤ 6 years with a suspected diagnosis of NF1, considering that the disease is virtually 100% penetrant at 6 years of age.

Design During this 10 year prospective multidisciplinary follow-up study, 209 children suspected of NF1 were examined; 150 were diagnosed with NF1; 59 were not. The present analysis included children in whom NF1 was considered present at 6 years of age (n = 85) and children without NF1 at 6 years of age (n = 42).

Results The minor disease features macrocephaly (52.9%), short stature (24.7%), hypertelorism (63.5%) and thorax abnormalities (37.6%) were highly prevalent in NF1-affected children and significantly associated with a diagnosis of NF1 at 6 years of age. In addition, the mean number of minor disease features was significantly higher in children with NF1 at 6 years of age compared to the group without a diagnosis at 6 years of age (mean 1.8 versus 0.8; p-value < 0.001). Moreover, children with three or more minor disease features were all diagnosed with NF1 under the age of 6 years. Multivariate analysis using a logistic regression model showed that macrocephaly, short stature, hypertelorism and thorax abnormalities were all independently associated with the presence of NF1 at 6 years of age.

Conclusion In children with insufficient diagnostic criteria and ≤ 6 years of age, documentation of minor disease features may be a helpful aid in predicting the diagnosis of NF1 in years to come.

Introduction

Type 1 Neurofibromatosis, NF1, is a common genetic disorder with an autosomal dominant inheritance. Half of all patients represent sporadic cases. Diagnostic criteria were established by the National Institutes of Health (NIH) and two or more criteria must be present for the diagnosis. In cases, in which a first degree family member is affected, only one additional criterion is sufficient for a diagnosis (1). The penetrance of the disease is regarded as virtually 100% at 5 years of age (2) and the diagnostic criteria have been shown to be applicable in children from 6 years of age (3).

Although the NF1 gene was cloned in 1990 (4-6), mutations in this large and complex gene are detected in only 10-20% of NF1-patients using classical methods such as polymorphic markers, single strand conformational analysis (SSCP) and Southern analysis on DNA from lymphocytes (7). Accordingly, mutation analysis is not available as a diagnostic tool, and in the majority of NF1-patients a diagnosis will still be based on the presence of two or more clinical criteria.

In addition to the diagnostic signs, physical characteristics such as macrocephaly, short stature, hypertelorism and thorax abnormalities are observed more frequently in NF1-patients than in the general population. As such, these characteristics are considered minor disease features (8).

In this study, we aimed to assess the frequency of minor disease features in children with NF1 and to evaluate the association of the presence of minor disease features with a diagnosis of NF1 in children at 6 years of age. We hypothesized that in contrast to the diagnostic criteria which develop with age, macrocephaly, short stature, hypertelorism and thorax abnormalities are present from birth and therefore may be of value in predicting NF1 diagnosis. In this way, parents of children suspected of NF1 and younger than 6 years of age can be informed of the necessity of diagnostic follow-up and both parents and children may benefit from the knowledge that many of the child's symptoms are due to an underlying disorder.

Patients and Methods

Study population
Between July 1985 and January 1996, a multidisciplinary NF1-team in the Sophia Children's University Hospital in Rotterdam including a pediatrician, dermatologist, pediatric neurologist, ophthalmologist and clinical geneticist, evaluated children suspected of having NF1. Children were referred with a suspected diagnosis of NF1 by general practitioners and medical specialists. Age at referral was variable; mean age at first examination date was 5.3 years. Diagnosis was based on the NIH criteria, assuming full penetrance of NF1 at 6 years.

Follow-up examinations were performed regularly according to a standardized protocol. Initial assessment included patient history (e.g. age, sex, type of schooling and presence of additional support by speech therapist or physical therapist), physical examination (e.g. length, weight, headcircumference, presence of dysmorphic features) and motor coordination tests supplemented by additional diagnostic tests if required. (9).

Minor disease features

The following physical features were defined as minor disease features and may be considered as present from birth. Macrocephaly is a head circumference equal to or exceeding the 97th percentile (specified for age and sex), irrespective of other body measurements and head circumference of family members. Children diagnosed with hydrocephaly were excluded from this group. Short stature is defined as a height equal to or below the third percentile (specified for age and sex), irrespective of other body measurements and height of family members. Children with NF1 and endocrinological disorders leading to short stature were excluded (n = 6). Hypertelorism was recorded at clinical assessment by an experienced pediatrician (clinical impression of hypertelorism). Inter- and outer canthal distances were not measured. Thorax abnormalities scored included both pectus excavatum and pectus carinatum.

Other minor disease features such as learning difficulties, speech abnormalities, behavioral- and motor problems were not analyzed due to lack of objective standards.

Analyses

In order to assess the frequency of minor disease features in children with NF1 and to evaluate the association of the presence of minor disease features with the presence and absence of NF1 in children at 6 years of age, two groups were selected from the total population. The first group (group 1) consisted of children diagnosed with NF1 at or before the age of 6 years. The second group (group 2) included children without a diagnosis of NF1 at 6 years of age. All children were initially suspected of the disease when they were younger than 6 years of age.

All data were registered on a standardized form and analyzed using SPSS 6.0. In univariate analyses, associations between the presence or absence of minor disease features and NF1 diagnosis were assessed using Fisher's exact test. Two-sided p-values were calculated at a significance level of 0.05. The number of minor disease features was computed in both patient groups and a difference in the mean number was tested for significance using the t-test for independent samples.

In addition, multivariate analyses were performed in order to assess the independent value of each minor disease feature in the diagnosis of NF1, using a logistic regression model. Variables significant in the univariate analysis (p-value < 0.05) were included in the logistic regression model. Variables were excluded from the multivariate model on the basis of the likelihood ratio test if the p-value was lower than < 0.05.

Results

Patients

During this prospective 10 year follow-up study, 209 children suspected of NF1 were examined; 150 were eventually diagnosed with NF1; 59 were not (prevalence of NF1 in children referred to the clinic: 71.8%). In 85 (49 boys, 36 girls) of the 150 children with NF1 the disease was diagnosed at or before 6 years of age (group 1). The age at diagnosis of the remaining 65 children was > 6 years due to diagnostic delay. Forty-two (26 boys, 16 girls) of the 59 children did not have NF1 at 6 years of age (group 2) (table 3.1). The remaining 17 children without NF1 were younger than 6 years of age and could therefore still develop NF1. Hence, they were excluded from the present analysis as we focused on children without NF1 diagnosis. Age at diagnosis was dependent on age at presentation of symptoms and on age at referral. Children without an NF1-diagnosis at the end of follow-up, appeared to be initially referred to the clinic on the basis of a positive family history for NF1 or had insufficient diagnostic criteria and minor disease features. In 14 of these 42 children (33.3%) one diagnostic criterion other than a positive family history was present; in 28 of these 42 children (66.7%) no diagnostic criteria other than a positive family history were present.

Table 3.1
Age distribution at diagnosis
within groups with NF1 (n = 150) and without NF1 (n = 59)

	NF + n = 150	NF - n = 59
≤ 6 years	85 [1]	17
> 6 years	65	42 [2]

Patients in shaded cells were selected for this analysis;
[1] = **Group 1**; [2] = **Group 2**

Univariate analysis

Table 3.2 presents the frequencies of minor disease features in patients with and without NF1 at 6 years of age. Macrocephaly was present in 52.9% of NF1-affected children, short stature in 24.7%, hypertelorism in 63.5% and thorax abnormalities in 37.6%. Comparison of the frequencies of minor disease features between both patient groups, showed that macrocephaly, short stature, hypertelorism and thorax abnormalities were significantly associated with the presence of NF1 at 6 years of age (table 3.2).

Table 3.2

Associations between minor disease features in children with and without NF1 at 6 years of age

Characteristics	Group 1 NF1 + n = 85	Group 2 NF1 - n = 42	Rate difference (95% CI)	p-value
Sex (% males)	42.4	38.1	(-0.22; 0.143)	0.093
Positive family history (%)	52.9	52.4	(-0.19; 0.18)	0.953
Mean age at first examination (yrs)	3.9	7.7	(-5.25; -2.36)	< 0.001 *
Mean age at last examination (yrs)	8.3	10.8	(-3.99; -0.97)	0.001 *
Mean follow-up time (yrs)	4.4	3.1	(-0.11; 2.79)	0.070
Macrocephaly (%)	52.9	31.0	(-0.40; -0.04)	0.017 *
Short stature (%)	24.7	4.8	(-0.31; -0.85)	0.001 *
Hypertelorism (%)	63.5	31.0	(-0.50; -0.15)	< 0.001 *
Thorax abnormality (%)	37.6	9.5	(-0.42; -0.14)	< 0.001 *

CI = confidence interval; * significant at level $p \leq 0.05$

Hypothetically, if the associations between minor disease features and a diagnosis of NF1 at 6 years of age should be due to an excess of minor disease features in children with NF1 and aged ≤ 3 years at the time of diagnosis, the additional value of minor disease features in diagnosing children ≤ 6 years of age would be minimal. Therefore, subgroup analyses were performed excluding children diagnosed at ≤ 3 years of age. Although sample numbers decreased, results were similar. The association between minor disease features and the presence of NF1 was still significant (data not shown).

Table 3.3
*Number of minor disease features (%) in children
with and without NF1 at 6 years of age*

Number of minor disease features	Group 1 NF $^+$ n = 85; %	Group 2 NF1 $^-$ n = 42; %
0	11 (12.9)	18 (42.8)
1	17 (20.0)	16 (38.1)
2	38 (44.7)	8 (19.1)
3	17 (20.0)	-
4	2 (2.4)	-

t-test for independent samples; p-value < 0.001

The number of children with multiple minor disease features was significantly higher in group 1 as compared to group 2. The mean number of minor disease features was 1.8 and 0.8 respectively (table 3.3). Children with three or more minor disease features were all diagnosed with NF1 under the age of 6 years. The cumulative percentages of children with two or more minor disease features were also significantly higher among patients in group 1 (67.1%) compared to group 2 (19.1%). In addition, 12.9% of children had no minor disease features in group 1, compared to 42.8% of children in group 2. This difference was also highly significant (Fisher exact test; p-value < 0.001).

Multivariate analysis
Table 3.4 shows the results of the multivariate logistic regression analysis. Each minor disease feature was independently associated with the presence of NF1 at 6 years of age. Odds ratios varied from 2.1 for macrocephaly to 8.0 for short stature. Short stature (OR = 8.0) and thorax abnormalities (OR = 5.7) were the most important independent predictors of the presence of NF1 at 6 years of age.

For the same reasons as mentioned above, we constructed a logistic regression model excluding children diagnosed ≤ 3 years of age. Odds ratios were similar to those computed in the overall analysis although confidence intervals were wider as expected.

Table 3.4
*Relative risk (95% CI) according to presence or absence of minor disease features
for the eventual diagnosis of NF1 in children ≤ 6 years of age at presentation*

Variables	β	OR	95% CI
Constant	-10.6		
Macrocephaly	0.73	2.1	(1.19; 2.97)
Short stature	2.08	8.0	(6.45; 9.59)
Hypertelorism	1.15	3.1	(2.26; 4.03)
Thorax abnormality	1.73	5.7	(4.42; 6.90)

CI = confidence interval

Discussion

The minor disease features macrocephaly, short stature, hypertelorism and thorax abnormalities are very common in NF1-affected children ranging from 24.7% for short stature to 63.5% for hypertelorism. A significant association was found between their presence and a diagnosis of NF1 at 6 years of age. Furthermore, the mean number of minor disease features in children diagnosed with NF1 at 6 years of age is significantly higher than in children without NF1 at 6 years of age. Multivariate analyses showed odds ratios varying from 2.1 for macrocephaly to 8.0 for short stature, confirming an independent association between each variable and NF1 diagnosis at 6 years of age. The highest odds ratios were observed for short stature (8.0) and thorax abnormalities (5.7).

Our findings suggest that, in the practice of a multidisciplinary clinic, the probability of developing NF1 in the presence of insufficient diagnostic criteria may be predicted on the basis of minor disease features. We have attempted to quantify this probability in order to define a group of children in which follow-up is essential, as the risk of developing NF1 is substantial. In this way, parents of children suspected of NF1 and younger than 6 years of age can be informed of the necessity of diagnostic follow-up and both parents and children may benefit from the knowledge that many of the child's symptoms are due to an underlying disorder.

Minor disease features could be of special importance to children with a de novo mutation in the NF1 gene as they may be diagnosed at a later age than children with an affected relative.

The importance and applicability of the diagnostic criteria in children is generally accepted. Korf et al. showed that 24 of 41 (58%) children went on to develop NF1 according to NIH criteria, after an initial visit during which only *café-au-lait* spots were observed. Almost all children in this study were diagnosed within three years of follow-up and before five years of age (10). Obringer et al. classified 151 out of 160 children (94%) under 6 years of age on the basis of the diagnostic criteria, including 80% of children with a negative family history (3). We emphasize that minor disease features should only be used to predict the likelihood of NF1 diagnosis when children have been checked thoroughly for the presence of conventional diagnostic criteria by NF1-specialists.

In the Welsh study, similar prevalences were reported of macrocephaly (45%) and short stature (31.5%) (11). Riccardi also reports short stature (more than one third below fifth percentile) and pectus excavatum (31%) to be present in a high percentage of his patients (12). As such, the association between minor disease features and NF1 is not surprising. However, the possible value of these variables in diagnosing NF1 has so far not been discussed.

We realize that the group of children without NF1 at 6 years of age, may still contain children with an NF1 gene mutation. Particularly, children with one diagnostic criterion other than a positive family history in combination with several minor disease features remain suspect (13). In literature atypical NF1-patients have been described with reduced penetrance(14) or late expression of the disease (11) In addition, cases of germline and somatic mosaicism have been reported (15-17). Follow-up of the children not diagnosed with NF1 at 6 years of age may result in additional diagnoses. Nevertheless, associations between minor disease features and a diagnosis of NF1 at 6 years of age would not be influenced as omittance of these cases would only make the association stronger.

The data on hypertelorism are crude as we did not use physical measurements. Therefore we recommend validation of the associations and the logistic model in a similar setting, in which hypertelorism is measured. Furthermore, although children were referred suspected of NF1, it is possible that children were referred on the basis of insufficient diagnostic criteria and minor disease features, leading to an underestimation of the odds ratios. In contrast, an overestimation may have been made due to referral at a later age, with insufficient diagnostic criteria and less minor disease features.

Easton presented evidence that modifying genes may play a major role in the variability of NF1 within families (18). Presumably, modifying genes may also be of influence on the presence of minor disease features in NF1-patients. In time, minor disease features may be of importance in discovering loci for these modifying genes.

As mutation analysis becomes more important in diagnosing (atypical) NF1-patients, minor disease features may also gain importance in isolating those (atypical) patients suspected of an NF1 gene mutation. It remains odd that a positive family history in a child with a reduced expression of the disease and > 6 years, will lead to a diagnosis and the same expression in a child (> 6 years) without affected family members will not. In the future, more clarity may even be won by excluding a positive family history from the diagnostic criteria for NF1. Basically, disease mechanisms are dependent on the gene mutation, synthesis of mRNA and protein, the genetic background and environmental factors and independent of a positive family history.

Other diagnoses than NF1 should always be considered in children with insufficient diagnostic criteria for NF1. In our study one of the 14 children > 6 years, with only one (clinical) diagnostic criterion was diagnosed with Neurofibromatosis type 2 during follow-up (dermal neurofibromas, bilateral vestibular schwannomas), one child was diagnosed with Leopard syndrome and a third child was diagnosed with Proteus syndrome. No minor disease features were present in these three children. No alternative diagnoses could be made in the remaining children. Other studies include Bannayan-Riley-Ruvalcaba syndrome, polyostotic fibrous dysplasia, McCune Albright's syndrome, urticaria pigmentosa, multiple lipomas, congenital generalized fibromatosis and steatocystoma multiplex in the differential diagnosis (8, 12).

In conclusion, in children with insufficient diagnostic criteria, under the age of 6 years and examined in a multidisciplinary NF1-clinic, documentation of minor disease features may be a helpful aid in setting a diagnosis NF1.

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Chapter 4

Endocrinological disorders and optic pathway gliomas in children with Neurofibromatosis type 1

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Abstract

Objective To establish the prevalence of endocrinologic disorders in children with Neurofibromatosis type 1 (NF1) and the relationship between these disorders and cerebral abnormalities on magnetic resonance imaging.

Design A prospective follow-up study.

Setting A multidisciplinary Neurofibromatosis clinic.

Patients A total of 122 children diagnosed with NF1 according to diagnostic criteria set by the National Institutes of Health.

Results Central precocious puberty (CPP) was diagnosed in 3 children and growth hormone deficiency (GHD) in 3 children. Optic pathway gliomas were observed in 15 children; in 9 of the 15 cases, the optic chiasm was involved. Of the 3 children with CPP, only 1 showed a chiasma glioma on magnetic resonance imaging. In 1 case with GHD an optic chiasm glioma was detected on neuroimaging. Two of the 9 children with an optic chiasm glioma presented with CPP or GHD.

Conclusions It has been suggested that CPP in children with NF1 is found exclusively in the presence of a chiasma glioma. We conclude that chiasma glioma may not be obligatory in children with NF1 and CPP or GHD. Moreover, we report a prevalence of GHD in children with NF1 of 2.5% which has not been established earlier.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal-dominant disorder affecting 1 in 3300 individuals. Characteristic features are *café-au-lait* spots, freckling in intertriginous regions, neurofibromas, and Lisch nodules (iris hamartomas). Diagnostic criteria have been established by National Institutes of Health (NIH) (1).

Endocrine disorders have been reported in 1% to 3% of all NF1-patients. In adults, pheochromocytoma is the most common disorder, presenting in 1% of NF1-patients (2). In children with NF1, central precocious puberty (CPP) is the most frequent endocrinopathy and is

seen in 3% of NF1-patients compared with 0.06% in the general pediatric population (3-5). Although various case reports have been published, the prevalence of growth hormone deficiency (GHD) in children with NF1 is unknown and is assessed in this paper.

Listernick et al. (6) reported that routine magnetic resonance neuroimaging in 176 children with NF1, revealed optic pathway gliomas in 33 (19%). Almost half of these gliomas were asymptomatic. In particular, gliomas of the optic chiasm have been reported to cause endocrinologic disorders, especially CPP (3).

In a prospective follow-up study, in a multidisciplinary NF1 clinic, we analyzed the prevalence of endocrinologic disorders in children and the relationship between these disorders and cerebral abnormalities on neuroimaging.

Patients and Methods

Since 1985, a multidisciplinary NF1 team at Sophia Children's University Hospital in Rotterdam (including a pediatrician, dermatologist, pediatric neurologist, ophthalmologist, and clinical geneticist) has evaluated children suspected of having NF1 according to NIH criteria. Besides periodic clinical evaluations, x-rays of the cranium and the entire spine and a visual evoked potential (VEP) are performed at presentation. Computed tomography (CT) scanning and/or magnetic resonance imaging is initiated when indicated.

Before January 1995, 171 new cases were seen. NF1 was diagnosed in 122 children (58 girls, 64 boys). The average follow-up period in the NF1-affected group was 4.4 years (standard deviation (SD) = 4.1). The mean age of these children at last examination date was 9.9 years (SD = 5.3). Still at risk for CPP were 16 of 58 (28%) girls who were < 8 years of age and 25 of 64 (39%) boys < 9 years at last examination date. Of the remaining 49 children, 17 remained suspect for NF1, having only one diagnostic criterion, other than a positive family history. In 32 children the diagnosis of NF1 was rejected.

CPP was defined as the appearance of secondary sexual characteristics before the age of 8 years in girls and 9 years in boys. Secondary sexual characteristics included breast development of at least Tanner stage B2 in girls and genital development of at least Tanner stage G2 and a testicular volume of ≥ 4 mL in boys (7). In addition, increased growth velocity, accelerated bone maturation, and pubertal responses of plasma luteinizing hormone (> 10 mU/L) during a

standard luteinizing hormone-releasing hormone (LHRH) stimulation test were present in all cases (8, 9). Pituitary gonadotropin response to LHRH was obtained after intravenous injection of 100 μg of LHRH. Subsequently, serum samples were taken at 0, 30, and 60 minutes after injection (10).

The diagnosis of GHD was based on a decreased height velocity over a period of at least 6 months or a decrease in height SD score of > 0.25/year (11). In addition, maximum plasma growth hormone (GH) peaks remained below 20 mU/L in at least two standard pharmacologic provocation tests (clonidine, arginine). After administration of 0.15 mg/m^2 clonidine orally or 0.5 g/kg body # weight arginine solution intravenously in 30 minutes, GH plasma values were measured at 0, 30, 60, 90, 120, and 150 minutes for the clonidine test, and at 0, 15, 30, 75, 90, and 120 minutes for the arginine test. Plasma GH was determined by a specific radioimmunoassay, using polyclonal antibodies after acid chromatography (12). Prepubertal girls were estrogen primed from the age of 8 years, prepubertal boys from the age of 9 years. A plasma insulin-like growth factor 1 value of 2 SDs below the reference values for age supported a diagnosis of GHD (12). Other causes of decreased growth velocity were excluded.

Thyroid hormones were evaluated in all patients suspected of having an endocrinologic abnormality; no abnormalities were detected. Bone ages were analyzed according to Greulich and Pyle. When bone age and chronological age differed > 1 year, bone age was considered divergent.

In our population, CT and MRI of the brain were performed only on indication. Indications included endocrinologic disorders; ophthalmologic abnormalities, such as optic atrophy, a relative afferent pupillary defect, an abnormal Ishihara color vision test, or an abnormal VEP; mental retardation; and various other neurologic abnormalities. In all patients suspected of having an optic pathway glioma, a special protocol for the detection of these gliomas was used (13). All patients with an endocrinologic disorder were evaluated by MRI.

CPP patients were treated with the LHRH analogue, Triptoreline (Ferring, Hoofddorp), a depot preparation, once every 4 weeks intramuscularly. In GHD patients, treatment was initiated with recombinant human GH.

Children who developed endocrinologic abnormalities secondary to cranial irradiation for a progressive optic pathway glioma or malignancy were excluded from this study.

The study protocol was approved by the medical ethics committee of the Medical Faculty and the University Hospital Sophia/Dijkzigt.

Data were analyzed using Dbase IV and SPSS (Statistical Products and Services Inc., Chicago, IL).

Results

Endocrinologic disorders were diagnosed in 6 of the 122 children (5.0%; 95% CI: 0.3%-9.7%) children with definite NF1. All patients were boys. CPP was diagnosed in 3 (2.5%) of the 122 children (table 4.1). Mean age at diagnosis was 7.3 years. GHD was detected in 3 (2.5%) of the 122 children (table 4.1). The mean age at diagnosis was 9 years. In 5 of the 6 children, NF1 was also diagnosed in one of the parents (3 mothers, 2 fathers).

In children diagnosed with CPP, a chiasma glioma was seen on MRI in one child (patient A). Patients B and C showed no optic pathway glioma on MRI and had normal VEP, ophthalmologic, and neurologic examinations (table 4.1). Furthermore, a chiasma glioma was observed in one child (patient D) diagnosed with GHD (table 4.1).

Although no routine neuroimaging studies were performed, optic pathway gliomas were reported in 15 of 122 children with NF1 (12.3%; 95% CI: 6.5%-18.1%); 6 of the 15 (40%) were located in the optic nerve and in 9 of the 15 (60%) the optic chiasm was involved. In 5 of the 15 (33.3%) children with an optic pathway glioma, no ophthalmologic abnormalities (vision and fundoscopic examinations) were observed; in 14 of the 15 (93.3%) VEPs were abnormal. Endocrinologic disorders developed in 2 of the 9 children (22.2%; 95% CI: 10.2%-34.2%) with an optic chiasm glioma; no endocrine disorders were seen in children with an optic nerve glioma.

In total, 3 of 15 children (20%) were treated with radiotherapy for a progressive optic pathway glioma (mean age: 9.7 years); 2 of the 3 (66.6%) went on to develop GHD (patients G and H), although patient H developed this complication after the follow-up period. In 1 other child (patient I) both GHD and CPP developed after radiotherapy of the cranium for an astrocytoma. These 3 children were not included in this study.

Table 4.1
Diagnosis of CPP and GHD in Children With NF1

Central Precocious Puberty (CPP)	A	B	C
Optic pathway glioma [1]	Optic nerve and chiasm	-	-
Ophthalmologic investigation [2]	A	N	N
Sex	m	m	m
Age at diagnosis NF1 (yr)	6.2	7.5	4.8
Age at presentation of secondary sexual characteristics (yr)	8.5	7.5	7.6
Increased growth velocity	+	+	+
Accelerated bone maturation	+	+	+
Maximal LH response to LHRH stimulation (100 mU/L i.v.) (prepubertal response < 10 mU/L)	18.7	53.0	14.1

Growth Hormone Deficiency (GHD)	D	E	F
Optic pathway glioma [1]	optic chiasm	-	-
Ophthalmologic investigation [2]	A	A [3]	N
Sex	m	m	m
Age at diagnosis NF1 (yr)	5.0	2.7	5.0
Age at diagnosis GHD (yr)	8.6	11.3	7.0
Progressive deviation from growth curve	+	+	+
Delayed bone maturation	+	+	+
Maximum plasma GH value after provocation (mU/L)			
arginine test (normal: >20mU/L)	11	8.5	13
clonidine test (normal: > 20 mU/L)	15	5.5	15
Plasma IGF-1 (< -2 SD)	+	+	+

+ = present; - = absent; N = normal; A = abnormal; m = male
[1] All patients screened by MRI, using a special protocol for detection of optic gliomas.
[2] Vision, fundoscopy, visual field examination.
[3] Hydrocephalus due to aqueduct stenosis caused papilloedema, which disappeared after treatment for hydrocephalus.

Discussion

The prevalence of GHD in children with NF1 was 2.5%, significantly higher than the 0.03% observed in the general pediatric population (14). In 1 of the 3 children with GHD, a chiasma glioma was detected; 1 of the 9 (11.1%) children with a chiasma glioma presented with GHD. In our 3 cases, no growth of neurofibromas that could be ascribed to GH therapy, was observed during clinical follow-up.

In our study, the prevalence of CPP is similar to the 3% observed by Habiby et al. (3) (table 4.2). In contrast, we did not find evidence that optic chiasm glioma is a prerequisite for CPP, because only one of three children with CPP had an optic chiasm glioma at the time of diagnosis. This is the first observation demonstrating that a chiasma glioma can be absent in the presence of CPP, using MRI.

Strikingly, in our study all endocrinologic disorders were observed in boys. In the literature, CPP is reported 8 times more often in girls than in boys and is sporadic and idiopathic in most cases (15). However, underlying organic disorders are more commonly discovered in male patients. Habiby et al. (3) found a female:male ratio of 2:5 in children with NF1 and CPP, as opposed to a female:male ratio of 22:11 in children with an optic pathway glioma. It appears that being a male with NF1 somehow predisposes one to CPP without an apparent explanation.

Saxena (16) was the first to report precocious puberty as well as marked growth retardation in children with NF1. Various case reports published later on endocrine manifestations in NF1 (17-20) concerned primarily CPP (table 4.2), not GHD. Comparison of the studies is difficult because of the lack of clinical data as well as the fact that diagnostic criteria for NF1 were only defined by NIH in 1987. In addition, detection of optic pathway glioma with neuroimaging became more sensitive with the introduction of MRI. Generally, it was thought that all endocrinologic disorders in NF1 could be attributed to central nervous system tumors, such as chiasmal optic gliomas, compromising hypothalamic and pituitary function (2, 21). In a study comparing the clinical manifestations and natural history of optic pathway gliomas in children with NF1 compared with children without NF1, Listernick et al. (22) reported CPP in 5 of 17 children with an optic glioma and NF1, in contrast to no cases of CPP in a group of children with an optic pathway glioma and no features of NF1. The latter group appears predisposed toward endocrinologic complications, because chiasmal involvement was significantly more common. This seems to suggest an association between CPP and NF1 itself, which cannot be attributed solely to the presence of a chiasma glioma. Our data support this association.

Table 4.2

Studies on Endocrine Manifestations of NF1

Author/ ref (year)	Neuroimaging technique	Number of children	Reason of referral	Results
Saxena [16] (1970)	PEG X-ray skull	5	Growth/ endocrine disorders	NF1, intracranial mass in 1/5 patients
Tertsch [17] (1979)	PEG X-ray skull/ optic foramina	1	Premature menstruation	NF1, chiasma glioma
Iraci [18] (1980)	PEG X-ray skull/ optic foramina	24	OPG	3/24 (12.5%) children OPG develop CPP
Buonaguidi [19] (1982)	PEG CT	4	CPP	1/4 NF1 and OPG
Laue [20] (1985)	CT	7	CPP + OPG	4/7 children had NF1
Habiby [6] (1995)	MRI CT	219	NF1	7/219 (3%) CPP, all chiasma glioma 7/18 (39%) patients with chiasma glioma developed CPP
Cnossen [18] (1997)	MRI	122	NF1	3/122 (2.5%) CPP, 1/3 chiasma glioma 3/122 (2.5%) GHD, 1/3 chiasma glioma 2/9 (11%) patients with chiasma glioma developed CPP or GHD

PEG = pneumoencephalogram; NF1 = Neurofibromatosis type 1; OPG = optic pathway glioma; CPP = central precocious puberty; GHD = growth hormone deficiency; CT = computed tomography; MRI = magnetic resonance imaging

Possible causal mechanisms may be cerebral abnormalities still undetected at a neuroimaging level, such as slow-growing hamartomas. In 2 patients with CPP and 1 patient with GHD studied by T2-weighted MRI of the brain, high-signal-intensity foci were observed in the brainstem and supratentorial regions of the brain. These white-matter lesions are common in children with NF1 and frequently increase in size or number, later resolving as the children get older. (23). They are, however, not likely to be correlated with the development of CPP or GHD, because they are classically localized outside the sellar and suprasellar regions of the brain. Another possible explanation could be abnormalities at a cellular level. The latter is supported by current evidence that suggests that neurofibromin, the protein encoded by the NF1 gene, is part of a signal transduction chain extending from extracellular signals to transcriptional regulation in the nucleus (24).

Referral bias on the basis of endocrinologic abnormalities presumably may play a role in our prevalence of data of CPP and GHD in children with NF1. However, five of six children developed the endocrinologic disorder after diagnosis of NF1, during follow-up. In contrast, the last child was referred with enlarged testes and pubic hair at 8 years of age. The high number of familial cases of NF1 observed in this group of children may be attributable to an ascertainment bias, although approximately half of the NF1-patients examined were familial cases and half were sporadic cases. The disease was inherited from both mothers and fathers.

In conclusion, CPP and GHD are important complications in children with NF1. Although a glioma of the optic chiasm is often encountered in these patients, CPP and GHD also appear to develop in the absence of an optic chiasm glioma. Diagnostic follow-up is essential in children with NF1, because premature manifestations of puberty and deviations from the growth curve may be recognized. Subsequently, early treatment with LHRH analogues or recombinant human GH, respectively, can be initiated with possible attainment of target height. Continuous monitoring will also help to establish any possible side effects of these interventions.

Acknowledgements

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Chapter 5

Atlantoaxial dislocation
in a patient with Neurofibromatosis type 1;
A preoperative protocol

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Abstract

We present a case of atlantoaxial dislocation in a child with Neurofibromatosis type 1 and cervical dysplasias following dissection of a plexiform neurofibroma in the head/ neck region. The number of cervical vertebral complications associated with Neurofibromatosis type 1 and the perioperative risks of C1-C2 mobility makes this an important issue in the management of NF1-cases by the neurologist, neurosurgeon, radiologist and anesthesiologist.

Introduction

Neurofibromatosis type 1 (NF1), is a common genetic disorder with a prevalence of 1:3300 (1). Characteristic symptoms are *café-au-lait* spots, intertriginous freckling, Lisch nodules in the iris, neurofibromas, optic glioma and skeletal dysplasias (2). In the vertebral column scalloping of posterior vertebral bodies, enlarged intervertebral foramina are seen as well as defective pedicles, dysplasia of vertebral bodies and dural ectasias. Scoliosis is frequently observed. Intra- or paraspinal plexiform neurofibromas are sometimes associated with these defects, causing additional complications in NF1.

In a series of 144 children with NF1, diagnosed in this hospital since 1985, cervical spinal abnormalities were radiologically detected in 10% of 133 children, an incidence as reported by others (3). Of the 13 patients with cervical dysplasias five were operated. Postoperatively, one patient suffered from Horner syndrome, diaphragmatic paralysis, paresis of left arm and shoulder and an atlantoaxial dislocation.

Case report

This girl was diagnosed with NF1 according to National Institutes of Health (NIH) criteria (1) at one year of age, because of *café-au-lait* spots and freckling. At three years of age she presented with a left cervical plexiform neurofibroma (figure 5.1). Multiple dysplasias were detected in the cervical vertebral column (figure 5.2).

At three and a half years of age she presented with pain in the neck and progressive growth of the plexiform neurofibroma. Resection of the plexiform neurofibroma was performed. During operation under general anaesthesia with neuromuscular relaxation, the head was placed in
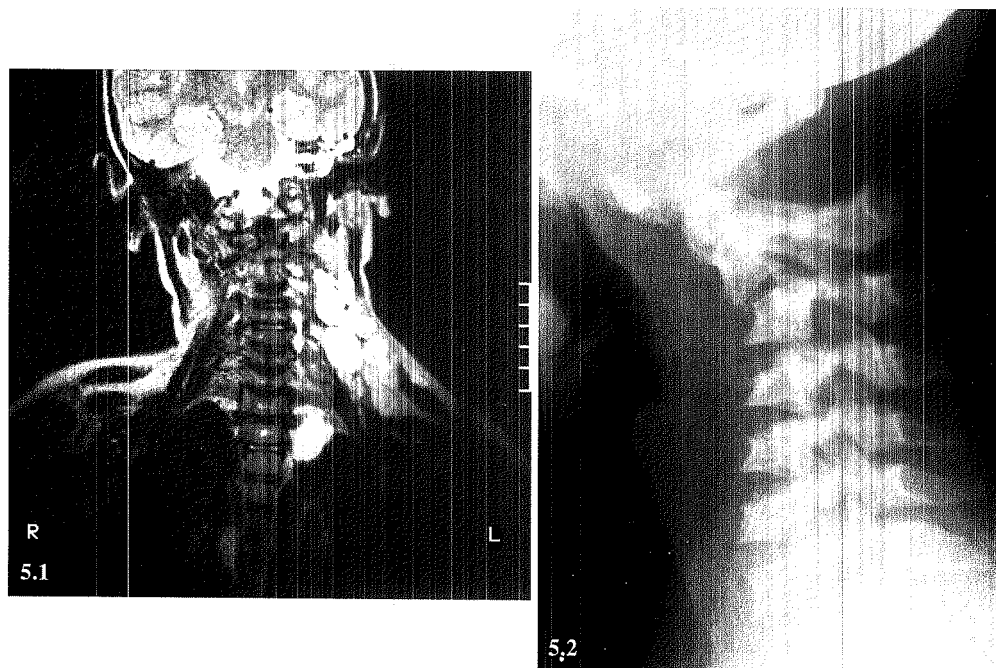
Figure 5.1
Preoperative MRI of the cervical vertebral column T2 weighted (TR 2280, TE 150) coronal image of the cervical spine. Plexiform neurofibroma is shown as a left-sided lobulated paravertebral mass with high signal intensity.

Figure 5.2
Postoperative X-ray of the cervical vertebral column. Lateral cervical spine during flexion. Increased distance between dens and C1 due to atlantoaxial instability. Abnormal shape of vertebral bodies C2-C5 and angular kyphosis at level C2-C3 is caused by bone dysplasia.

hyperextension to the right side. No abnormalities were observed at this time. Postoperatively, diaphragmatic paralysis, and paresis of left arm and shoulder due to a plexus brachialis lesion and Horner Syndrome were noted. The function of the arm and shoulder however, normalized in several weeks. Moreover, the child complained of prickling in both legs. Subsequent radiography and a flexion extension test of the cervical vertebral column showed an atlantoaxial instability with a ventral luxation of C1 (figure 5.2). A posterior cerclage was applied operatively between the first two cervical vertebrae to decompress the spinal cord, resulting in relief of the

paraesthesia's and an acceptable position of the atlantoaxial joint on radiograms. Histology showed no signs of malignant degeneration.

Discussion

Atlantoaxial dislocation is a severe complication of NF1 which should be borne in mind in each NF1-patient with cervical abnormalities undergoing general anaesthesia or other situations associated with traction and hyperextension to the cervical vertebral column. This complication is presumably caused by the increased mobility between C1-C2 due to either dysplasias of the cervical vertebral column, maldevelopment of ligaments, or the presence of a intra-/ paraspinal plexiform neurofibroma (4-7). We are the first to present a preoperative protocol for these at risk patients.

In our multidisciplinary Neurofibromatosis clinic, we now recommend the following, complete bidirectional cervical vertebral column X-rays in every NF1-patient undergoing anaesthesia and/ or surgery. If abnormalities are observed and intubation is required, a supplementary flexion-extension should be obtained to assess the perioperative risk. Subsequently, intubation should be performed under fixation of the cervical column or under fibrescopic guidance. The presence of cervical dysplasias or spinal neurofibromas may also be a reason to opt for regional anaesthesia if feasible.

The high prevalence of cervical vertebral complications in NF1-patients justifies a cautious approach towards NF1-patients undergoing anaesthesia and/ or surgery. We assume that this preoperative protocol will be helpful in preventing unnecessary risks in these patients.

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Chapter 6

Diagnostic delay in Neurofibromatosis type 1

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Abstract

*Since 1985 a multidisciplinary team in the Sophia Children's University Hospital in Rotterdam
provides diagnostic follow-up and genetic counseling services for NF1-patients and their families.*

*Parents of 68 affected children as well as 24 affected parents were interviewed. Half of the
affected children and one third of the affected adults were treated for symptoms related to NF1
before a specific diagnosis was made. Although the disease is fully penetrant by the age of five
years, 35% of the affected children had not been diagnosed by this age. Parents stated a
preference for early diagnosis of NF1. Diagnosis of NF1 did not seem to be a reason to refrain
from having children. The general attitude towards prenatal diagnosis was positive; however few
parents would actually terminate an affected pregnancy.*

Conclusion
*Overall delay in diagnosis of NF1 is significant. Knowledge of symptoms should make an early
diagnosis possible with beneficial effects for the patient and family members.*

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal-dominant inherited disorder, affecting 1:3300
newborn children. Half of all cases are caused by new mutations (1). Characteristic features of
the disease include *café-au-lait* spots, axillary freckling, neurofibromas and Lisch nodules. (2).
Severe complications are due to plexiform neurofibromas, optic gliomas (3), endocrinological
disorders (4), epilepsy, scoliosis, pseudoarthrosis (5) and mental retardation. Moreover there is
an increased risk of malignancies (6). Learning difficulties are observed in many children (7),
as well as a short stature, large head circumference, and hypertelorism (8). Symptoms are highly
variable and severity cannot be predicted, even within families. Generally one third of the
patients with the disorder are minimally affected, one third has mild to severe problems, and one
third is severely affected having one or more severe complications of the disease (9). The
penetrance of the disease is virtually 100% by the age of five years (10).

Since 1985 a multidisciplinary team including a pediatrician, a pediatric neurologist, dermato-
logist, ophthalmologist and clinical geneticist in the Sophia Children's University Hospital in

Rotterdam, provides diagnostic follow-up and genetic counseling services for NF1-patients and their families. The pediatrician is the coordinator of this follow-up program.

It seemed essential to analyze the time span between the development of symptoms and diagnosis of NF1 as a number of children visited the clinic with severe complications not recognized before as manifestations of NF1 by consulted clinicians. Other children were diagnosed with NF1 after a number of children with the disorder had been born into the family. In these families parents were often unaware that they were affected with an hereditary disease which could lead to severe complications in themselves and their children.

Therefore a study was carried out assessing diagnostic delay, the information provided at diagnosis about the disorder and its mode of inheritance, and the influence the diagnosis has had on family planning.

It has been suggested that NF1 may be underdiagnosed in children who are the first case in the family and who have no major disease complications (10). We report an overall delay of diagnosis in patients with NF1, which supports this hypothesis.

Patients and Methods

Questionnaires were sent to parents of 81 children with NF1 (age 2-22 years), from 70 families, visiting the NF1 follow-up program in the Sophia Children's University Hospital in Rotterdam since 1985. These children were diagnosed according to the criteria formulated by the National Institute of Health Consensus Development Conference in 1987 (table 6.1) (11). Questions were asked about the time span between symptoms and diagnosis, the attitude of the parents towards the diagnosis, the information given by the doctor at the time of diagnosis about the hereditary aspects of the disease, and the experiences of parents and children with the annual visits to the clinic. This questionnaire will be referred to as the "proband-questionnaire". Proband refers to a child with NF1 in this study, irrespective of an earlier occurrence of NF1 in the family. All parents could participate in this part of the study since this questionnaire focused on diagnosis of NF1 and the experiences parents had with the follow-up program in general.

Of the 81 questionnaires, 68 were sufficiently filled out for analysis of our population of NF1-patients (84%). The 68 children were from 58 families; each child was counted as one individual with NF1. An exception was made for questions in which the parent was likely to answer similarly in both children. Thirteen of the 81 questionnaires could not analyzed: 11 parents were not interested in participating, one couple refused to participate as their child had just been diagnosed with a malignant tumor and the guardian of another child was not acquainted with the child's medical history, as it lived in an orphanage. Probands were divided into a group of probable familial probands (n=37), if the child had an affected parent; and a group of probable single case probands (n=31), if this was not the case.

A similar questionnaire was sent to NF1-affected parents (24-55 years) of affected children in our follow-up program. Additional questions focused on decisions regarding reproduction. This questionnaire will be referred to as the "affected-parent's questionnaire". Initially, in 25 out of 70 families (36%) one of the parents was known to have the disease. The 25 affected parents accounted for 29 affected children. After genetic counseling the number of affected parents rose from 25 to 33 leading to the 37 probable familial probands. Of the 25 affected-parent's questionnaires 24 were sufficiently completed. Calculations were made using these 24 questionnaires (96%).

Of the 68 children 26 were female (38%) and 42 were male (62%). Eleven of the 24 affected parents were female (46%); 13 (54%) were male.

A supplementary telephone interview, in which 27 parents were reached (39%), was performed to analyze attitudes towards prenatal diagnosis of NF1, as questions on this subject were considered delicate. Sixteen of the adults were affected; 11 were unaffected with the disease.

The study protocol was approved by the Medical Ethics Committee of the University Hospital Sophia/Dijkzigt and conducted at the Sophia Children's Hospital. Informed consent was obtained from one or both parents of each subject.

Results were analyzed using Dbase IV and SPSS for personal computers (12).

Table 6.1
NIH Diagnostic criteria in NF1

2 or more of the following criteria are necessary for a diagnosis of NF1

Clinical signs

- 6 or more café-au-lait spots over 5 mm in greatest diameter in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals.

- Two or more neurofibromas of any type or one or more plexiform neurofibromas.

- Freckling in the axillary or inguinal region.

- Optic or chiasma glioma.

- Two or more Lisch nodules (iris hamartomas).

- A distinctive osseous lesion, such as sphenoid dysplasia or thinning of long bone cortex, with or without bowing and pseudoarthrosis.

Family History

- A first-degree relative (parent, sibling or offspring) with NF1 by the above criteria.

Results

Diagnosis

The average age at diagnosis was 4.5 years (range: 0-14 years; SE: .44) for the probands (figure 6.1) and 27.7 years (range: 7-41 years; SE: 1.8) for the affected parents (figure 6.2). Age at diagnosis in affected children and adults with the disorder differed significantly (T=-12.50; $p \leq 0.001$). Thirty five percent of the children had not been diagnosed by the age of five years. The majority of these children had major disease complications. No significant difference was found in age at diagnosis between familial case probands and single case probands. Table 6.2 shows that in more than half of the probands the diagnosis was established by a pediatrician and in one fourth by a dermatologist. Adults with NF1 were diagnosed in nearly half of the cases by a dermatologist and in one fifth by a general practitioner.

Diagnostic Delay

In 51% of the children and 38% of the adults early symptoms of the disorder were not immediately recognized as features of NF1 (table 6.3). Though retrospectively, symptoms of NF1, generally the *café-au-lait* spots, had been observed by the end of the first year in 77% of the children and in 67% of the adults. Patients were treated and/or referred to medical and

paramedical specialists. Table 6.4 shows that more than half of the probands and nearly half of the affected parents were treated by a pediatrician; almost one third of the probands and more than 40% of the affected parents by a general practitioner. Probands were referred to special learning programs in almost one third of the cases. The occurrence of symptoms requiring medical attention led to a concurrent diagnosis in 47% than half of the probands and almost 58% of the NF1 adults. When asked if parents would have preferred a diagnosis of NF1 before the occurrence of complications, 93% answered affirmatively. One parent (2%) had preferred a diagnosis when more severe symptoms appeared.

At the time of diagnosis of NF1, nearly one third of the probands did not have any serious symptoms of the disorder. Nevertheless, half of the parents of affected children found themselves relieved by a specific diagnosis in their individual case. Fifteen percent of the parents experienced the diagnosis as more of a burden than a relief. Thirty five percent of the parents did not answer this question.
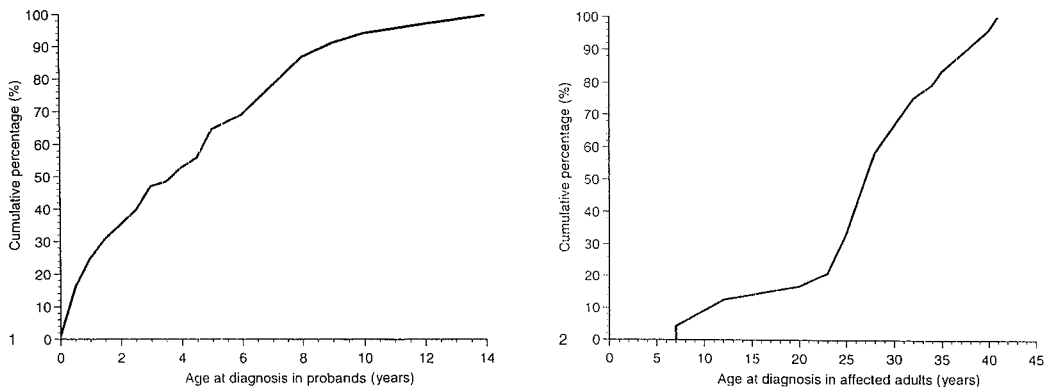

Figure 6.1
Cumulative percentage distribution of age at diagnosis in probands

Figure 6.2
Cumulative percentage distribution of age at diagnosis in affected adults

Table 6.2
Clinicians involved in primary diagnosis of NF1 in probands and adults [1]

| | Probands | | | | Affected adults | |
| | Probable familial case (n = 36) | | Probable single case (n = 31) | | (n = 24) | |
	n	%	n	%	n	%
General practitioner	O	0	1	3	5	21
Dermatologist	7	19	12	39	10	42
Pediatrician	27	73	13	42	2	8
Neurologist	3	8	6	19	3	13
Other fields [2]	8	22	7	23	5	21
Unknown	0	0	0	0	1	4

[1] As several alternatives were possible total percentages may exceed 100.0%.
[2] Ophthalmologist, dental surgeon, internist or schooldoctor.

Table 6.3
Treatment for NF1 symptoms prior to specific diagnosis of NF1

| | Probands (n = 68) | | Affected adults (n = 24) | |
	n	%	n	%
Medical intervention without NF1 diagnosis	35	51	9	38
Diagnosis at onset of symptoms	32	47	14	58
Unknown	1	2	1	4

Table 6.4
Medical interventions prior to diagnosis of NF1 [1] [2]

	Probands (n = 35)		Affected adults (n = 9)	
	n	%	n	%
General practitioner	10	29	4	44
Dermatologist	6	17	1	11
Pediatrician	19	54	4	44
Neurologist	3	9	2	22
Psychologist/ remedial teaching	10	29	0	0
Other fields [3]	2	6	1	11

[1]   n=35 and n=9 are both a subset of patients which received treatment prior to diagnosis of NF1.
[2]   As several alternatives were possible total percentages may exceed 100.0%.
[3]   ophthalmologist, physiotherapist, speech therapist, ENT doctor, orthopaedic surgeon.

Information

Thirty two percent of the parents of children with NF1 and 58% of the affected adults were not satisfied with the information provided at the time of the diagnosis. The most important sources of information at diagnosis for parents with a child with NF1 were a pediatrician (50%), a clinical geneticist (27%) and the patient support group (18%). One third of the affected adults found a clinical geneticist the most informative source, the patient support group and dermatologist were considered the most important source of information in one tenth of the cases.

The parents of children with NF1 and affected adults recollected being informed about the autosomal-dominant inheritance of the disease in 84% and 76% of the cases of which 13-21% were informed at the time of diagnosis. One patient was told about the heritability of the disease 41 years after diagnosis. Twelve percent of the parents and 25% of the affected adults could not recall being informed about the possibility of transmission of the disease.

In half of the probands and affected parents a clinical geneticist was found to be the most informative person regarding the hereditary aspect (table 6.5); for one third of the parents of probands and one fifth of the affected adults a pediatrician played an important role in explaining his aspect.

Examination of family members and children

Family members of NF1-patients were examined in 63-78% of the cases. Usually parents of the proband were examined (81%). Siblings of probands with NF1 were examined at the request of their parents in 31 of the 68 cases. Ten of these examined siblings (32%) came from single-case families.

Nearly half of the parents found the regular follow-up examinations burdensome for their child. The other half found examinations no problem at all. A few parents experienced the examinations of their child as a problem for themselves.

Table 6.5
Source of information on inheritance of NF1 [1]

	Probands (n = 68)		Affected adults (n = 24)	
	n	%	n	%
General practitioner	1	2	2	8
Dermatologist	5	7	3	13
Pediatrician	23	34	4	17
Neurologist	4	6	2	8
Clinical geneticist	31	46	13	54
Patient support group	5	7	0	0
> three of the above	1	2	0	0
Others [3]	3	4	1	4
Unknown	6	9	3	13

[1]   As several alternatives were possible total percentages may exceed 100.0%.
[2]   Ophthalmologist, physiotherapist, speech therapist, ENT doctor, orthopaedic surgeon.

Family Planning and Prenatal Diagnosis

Affected adults were asked whether having NF1 affected their decision to have children. At the time of diagnosis of NF1 13 of the 24 patients had planned to have (more) children. Four of them changed their mind thereafter and nine did not. Whether the couple had children did not influence their reproductive decision.

Twenty five of the 27 parents interviewed on their attitude towards prenatal diagnosis (93%) agreed in principle to prenatal diagnosis in pregnancies at risk for NF1; two parents were opposed to prenatal diagnosis. The latter group consisted of a father whose wife with NF1 had died following complications of the disease and one couple which had a child with a new mutation. Three parents would terminate an affected pregnancy, 12 were in doubt and 12 would not consider termination. There was no relationship between the severity of NF1 in the proband or affected parent, and choices after prenatal diagnosis.

Discussion

Diagnostic Delays

The age at diagnosis between adults affected with NF1 and affected children has decreased significantly. This is probably due to an increased awareness of the disease and its complications (9). One should also consider that the group of children with NF1 in our hospital has a higher prevalence of severe complications than can be observed in the population at large. This may bias the age at which NF1 is diagnosed. Although the disease is virtually fully penetrant at five years of age, 35% of the affected children in our study were not diagnosed with NF1 at this age. Half of the probands and more than one third of the affected adults were treated for symptoms of NF1 without being recognized as such by different physicians; though the hallmark of the disease, the *café-au-lait* spots was present in the majority of the cases before the end of the first year of life and could have formed an important clue in diagnosing the patient.

It has been (10) suggested that NF1 may be underdiagnosed in children, especially those who are the first case in the family and who have no major disease complications. Our data shows an overall delay of diagnosis of NF1, which supports this hypothesis. In the NF1-affected children and adults in which a diagnosis was eventually made, diagnostic delay was not significantly higher in sporadic cases in comparison to familiar cases; major disease complications were manifest in children with a significant diagnostic delay.

Adults were usually diagnosed by a dermatologist or general practitioner. The probands were generally diagnosed by a pediatrician. This is probably due to the fact that the children in our group were diagnosed at an earlier age.

Parents have clearly stated that they appreciate an early diagnosis of NF1 in their child even in the absence of severe symptoms (93%). When asked how they experienced the diagnosis at the time, half of the parents answered that they felt relieved in comparison to the insecurity they had

felt before diagnosis. Fifteen percent of the parents experienced the diagnosis of NF1 more as a burden than a relief. One third of the parents did not answer this question; this is exceptionally high and could be due to the fact that parents find the question too confronting or too difficult to answer.

An early diagnosis is usually preferred by parents as it resolves uncertainty about the cause of medical problems in the child and helps initiate interventions for previously unexplained learning, motor- and speech problems. Also, genetic counseling may be offered to the parents and other relatives. Careful information of parents of newly diagnosed NF1 cases is very important as to give a realistic perspective on the prognosis of NF1 and so prevent unnecessary anxiety.

Information and Inheritance

Information on NF1 can be optimalized as one third of the parents of probands and nearly two thirds of the affected adults were not satisfied with the information provided during various phases of the diagnostic process. The majority of the cases was informed about the autosomal-dominant mode of inheritance of the disease. Other studies have also observed a general dissatisfaction about the lack of information given at diagnosis (13). Patients are often informed on the inheritance of the disease, but the risk for future children cannot be stated with precision. Benjamin et al. (14) observed a significant difference between a group having had genetic counseling and one that had not, regarding information about the disease. In our study and other studies the clinical geneticist plays an important role in informing the patient about the clinical features of NF1 and clearly is the most important source of information on the mode of inheritance. Referral for genetic counseling seems essential for a better understanding of the disorder and its genetic aspects in afflicted families.

Surprisingly, only one fourth of the group recalled being informed about the inheritance directly at the time of diagnosis. The time span between these two points varied greatly: from almost zero to 41 years.

Reproductive Decisions

Many studies have shown that reproductive decisions are complex and involve factors other than the magnitude of the genetic risk and the severity of the disease (15, 16). In NF1 these reproductive decisions are complicated by the extreme variability of the disorder. The risk of passing on the disease is 50%, but only one third of NF1-patients develop severe complications requiring medical attention and the disease is rarely lethal.

In our study the diagnosis did not seem to be a reason to refrain from having children; The general attitude towards prenatal diagnosis was positive but few parents would actually terminate an affected pregnancy. The severity of the disease in family members or the parent did not have any effect on this decision. It should be considered that the predictive value of hypothetical reproductive decisions has been questioned (17). Experience with prenatal diagnosis in NF1 has taught us that very little use is made of this technique and when used, an affected pregnancy rarely leads to termination (18). Many studies concerning polycystic kidney disease and Huntington's Disease give similar results (15, 17, 19). The longer life expectancy of a patient with an autosomal dominant disorder, may affect the reluctance in opting for a termination of an affected pregnancy.

Conclusion

This article has shown that there is a significant overall delay in diagnosis of NF1. Knowledge of the symptoms makes an early diagnosis possible with beneficial effects for the patient as well as family members, as genetic counseling can be initiated. Contrary to what is generally believed, parents appreciate an early confrontation with the disorder and its complications.

Acknowledgments

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Chapter 7

Deletions spanning the Neurofibromatosis type 1 (NF1) gene: Implications for genotype-phenotype correlations in NF1?

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Abstract

Neurofibromatosis type 1 (NF1) is an autosomal-dominant disorder characterized by abnormalities of tissues predominantly derived from the neural crest. Symptoms are highly variable and severity cannot be predicted, even within families.

DNA of 84 unrelated patients with NF1, unselected for clinical features or severity, were screened with intragenic polymorphic repeat markers and by Southern analysis with cDNA probes. Deletions of the entire gene were detected in five patients from 4 unrelated families. Their phenotype resembled that of five previously reported patients with deletions, including intellectual impairment and dysmorphic features, but without an excessive number of dermal neurofibromas.

This report supports the hypothesis that large deletions spanning the entire NF1 gene may lead to a specific phenotype.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal-dominant inherited disorder affecting 1:3300 individuals. Half of all patients represent de novo mutations. Characteristic features include *café-au-lait* spots, axillary and inguinal freckling, neurofibromas and Lisch nodules (iris hamartomas). Diagnostic criteria have been established by the National Institutes of Health (NIH) (1). Symptoms are highly variable and severity cannot be predicted, even within families. The penetrance of the disease is virtually 100% by the age of 5 years (2).

The NF1 gene has been cloned and maps to chromosome 17q11.2. The gene spans a region of about 350 kb of genomic DNA, contains 60 exons, is ubiquitously expressed and encodes an 11-13 kb mRNA. Four alternative splice forms have been reported. The protein, neurofibromin, consists of 2,818 aminoacids and shows homology to the GAP-family (GTP-ase activating) of proteins in yeast and mammals. The NF1 gene presumably functions as a tumor suppressor gene (3).

After selecting 6 NF1-patients with dysmorphic facial features and mild to severe learning disabilities Kayes et al. detected deletions encompassing the NF1 gene in 5 patients (4). All these 5 patients had a large number of dermal neurofibromas.

We screened 84 unrelated NF1-patients from a pediatric and adult NF1-patient-set, irrespective of their phenotype, by microsatellite and Southern analysis. In 4 cases deletions spanning the entire NF1 gene were found; one of the probands had an affected parent, showing the same deletion. Extensive subsequent clinical examination revealed several common features, implying that genotype-phenotype correlations may be possible for some mutations in the NF1 gene.

Patients and Methods

Patients

DNA of 84 unrelated patients with NF1, diagnosed according the criteria of the NIH, were screened by microsatellite and Southern analysis. Patients were not selected for specific clinical features or severity of the disease and were referred to our clinic by physicians throughout the Netherlands. Ages of these patients varied from 2 years to 63 years of age with an average age of approximately 25 years. This study was approved by the Medical Ethical Committee of the University Hospital Sophia/Dijkzigt, Rotterdam.

Microsatellite and Southern analysis

DNA was isolated from peripheral blood according to standard procedures. Microsatellite analysis was performed by polymerase chain reaction amplification of the polymorphic intragenic repeat markers compoundrepeat in intron 26 (amplified fragment: 235-277 bp)(5), Alurepeat in intron 27 (395-407 bp)(6), CA repeat IVS27TG24.8 in intron 27 (270-292 bp), CA repeat IVS27AC28.4 in intron 27 (207-219 bp)(7), and CA repeat IVS38TG53.0 in intron 38 (171-187 bp) (8) (see figure 7.1). Additional intragenic polymorphic markers in exon 5 (9), exon 13 (10), intron 16 and intron 41 (11) and intron 39 (12) were tested in patient E. Fragments were separated on a 6% polyacrylamide gel. Paternity was tested using the polymorphic repeat markers HPRT (chromosome Xq26), FABP2 (# 4q28-q31), CD4 (# 12p12), CSF1R (# 5q33.3), THO1 (# 11p15.5), PLA2A (# 12q23-q ter).

For Southern analysis 6 microgram of genomic DNA was digested with the restriction enzymes *Bam*H1, *Hind*III, *Bgl*II, *Eco*RI, and *Taq*I, according to the manufacturer's instructions. Fragments were separated on a 0.7% agarose gel. After blotting on Hybond N+ membrane filters, these were hybridized with the overlapping cDNA probes GE2, AE25, FF13, P5 and B3A (figure 7.1). Hybridization and autoradiography were performed according to standard procedures.

When deletions extended beyond the NF1 gene, the boundaries of the deletion were determined by the extragenic probes beta8.2 (CRYB1), VAW215R3 (D17S120), VAW212R2i (D17S117), VAW210M1 (D17S115) and EW207 (D17S73) (figure 7.1).
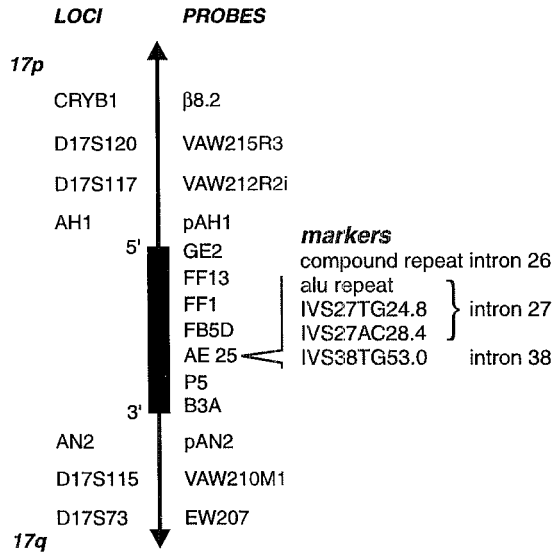

Figure 7.1
Localisation of NF1 cDNA probes, intragenic polymorphic markers and extragenic probes in the NF1 region, utilised in this study. Not drawn to scale.

Results

Microsatellite analysis and Southern analysis

In DNA of 84 unrelated and unselected NF1-patients microsatellite and Southern analysis were performed. Initially, polymorphic intragenic repeat markers showed a monoallelic pattern indicative for hemi- or homozygosity in a total of 6 patients. After Southern analysis, and microsatellite analysis in patients and their relatives we concluded that four of these patients were truly hemizygous for the NF1 gene based on decreased band densities for all cDNA probes (patient A, B, D, and E) and uniparental origin of polymorphic alleles in affected patients (A, B, and C).

The first two cases (patient A, patient B) were apparently sporadic cases. DNA of these patients was PCR-amplified using primers for the intragenic repeat markers compoundrepeat in intron

26, Alurepeat in intron 27, CA repeat IVS27TG24.8 in intron 27, CA repeat IVS27AC28.4 in intron 27 and CA repeat IVS38TG53.0 in intron 38. In patient A the marker IVS38TG53.0 was informative, showing absence of the maternal allele (figure 7.2). Patient B showed hemizygosity for the polymorphic compoundrepeat in intron 26, marker IVS27TG24.8 and marker IVS38-TG53.0, with loss of the paternal allele (data not shown). Upon analysis of DNA from patient C, who has an NF1 affected mother (patient D), it was shown that he lacked a maternal contribution when tested with the markers IVS27AC28.4, IVS27TG24.8 and IVS38TG53.0 (data not shown). We concluded that he had inherited a deletion from his mother. The origin of the deletion in the mother could not be established as no DNA of her parents was available.
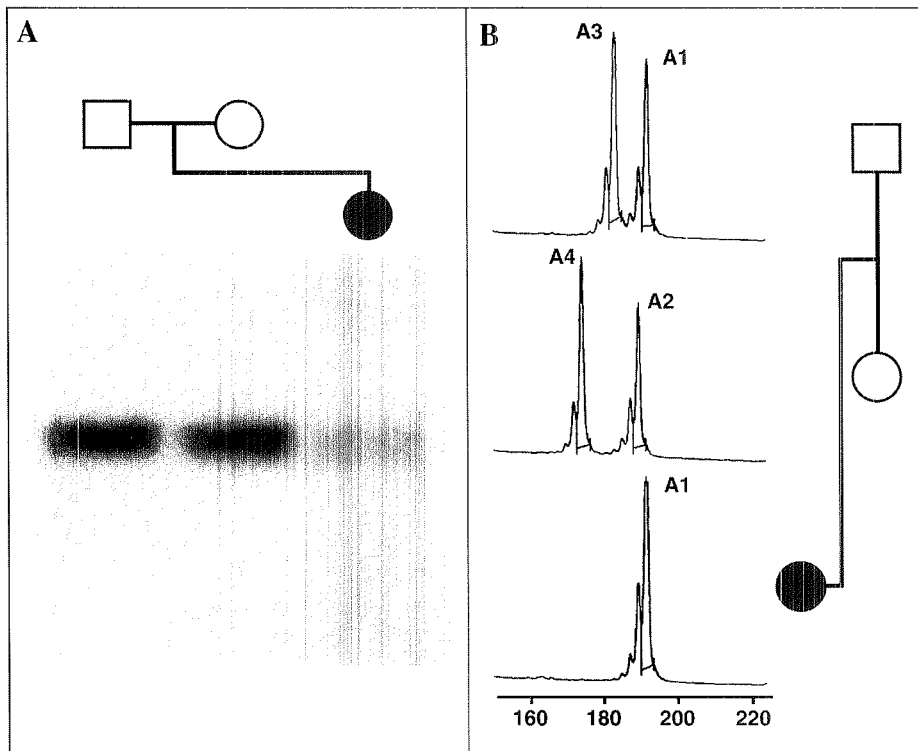
Figure 7.2

Absence of the maternal allele in sporadic patient (patient A) A. Southern blotting analysis using equalized amounts of DNA digested with XbaI and hybridized with cDNA probes B3A, showed a decreased intensity in the NF1-affected child. B. Intragenic repeat marker analysis with marker IVS38TG53.0. Father has alleles A1, A3; mother has alleles A2, A4; the affected child shows paternal allele A1 in the absence of a maternal allele.

None of the intragenic polymorphic markers (10 tested; see methods) were informative in family E. However DNA of patient E, repeatedly showed a reduced signal on Southern analysis for all intragenic cDNA probes. We concluded that this patient must also be hemizygous for the NF1 gene.

Non-paternity was excluded in all 4 cases with an NF1 gene deletion.

As all deletions extended beyond the boundaries of the NF1 gene, defined by the probes GE2 and B3A, we analyzed the length of the deletions with extragenic probes. The polymorphic marker D17S117 (VAW212R2i), flanking the 5' region of the gene, showed two alleles in all cases. We concluded that breakpoints of the deletions flanking the upstream region of the gene must be situated between D17S117 and cDNA probe GE2. Marker D17S115 (VAW210M1) located downstream of the NF1 gene showed two alleles in 3 of the 4 probands, indicating that the breakpoints of the deletions were situated between this marker and cDNA probe B3A. In the fourth proband (patient A) the deletion was shown to extend beyond marker D17S115, as the breakpoint was identified with marker D17S73 (EW207) after digestion with the restriction enzyme XbaI (figure 7.3). An additional fragment, that was not inherited from the parents, was present in DNA of the patient.
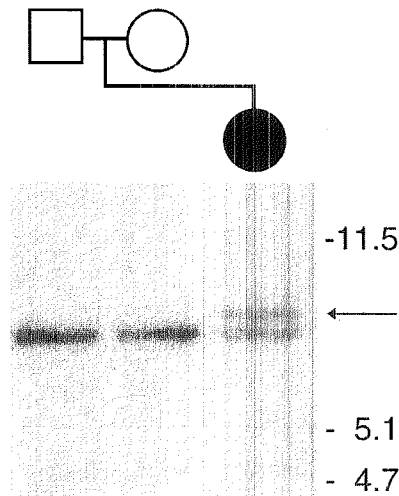

Figure 7.3
Characterization of the breakpoint in patient A in the region downstream of the NF1 gene. Southern blotting analysis of XbaI digested DNA of family A, hybridized with marker D17S73 (EW207). The arrow indcates the aberrant fragment in DNA of the indexpatient.

Clinical data

Of the 4 unrelated patients in which a deletion was found, three were sporadic cases (patient A, B, E) and one had an NF1-affected parent (patient C). The phenotype of the affected parent (patient D) has been included in the following data. All patients have been examined recently (table 7.1). Macrocephaly and hypertelorism were not included in the dysmorphic features as these are often seen in NF1-patients, without other dysmorphic characteristics.

Patient A, last examined at thirteen years of age, was diagnosed with NF1 at 12 years of age. She completed a normal elementary school with the aid of remedial teaching and now attends a school for the moderately mentally retarded. Her behavior was not appropriate for her age, her speech was unclear and her facial features showed hypertelorism, bilateral ptosis, an extreme overbite of the maxilla, with broad lips, very broad nose and slight webbing of the neck. The thorax showed mild pectus excavatum, hands and feet were broad and short, the arms showed cubiti valgi. Her symptoms, though suggestive for Noonan-like NF1, do not meet the criteria for the latter as postulated by Duncan et al. (13), a comprehensive scoring system for evaluating Noonan syndrome. Symptoms specific for NF1 included multiple *café-au-lait* spots, axillary freckling, and a few dermal neurofibromas.

Patient B, a girl of 11 years of age, attends a school for the mentally retarded. Besides retrognathia and deepset eyes, the left side of the face showed overgrowth compared to the right side. There was a decreased vision of the left eye, though an optic glioma could not be detected. The patient often complained of headaches and itching. Specific symptoms of NF1 were the *café-au-lait* spots, freckling and Lisch nodules. No dermal or plexiform neurofibroma were reported.

Patient C, a boy of 7 years of age, inherited the deletion from his mother (patient D). He attends a school for the mentally retarded. Besides macrocephaly and hypertelorism, epicanthal folds, prominent philtrum, large ears, slight micrognathia and postnatal overgrowth of the skeleton were observed. The postnatal overgrowth, also observed in the mother (patient D), suggested a possible Weaver syndrome. *Café-au-lait* spots, freckling and a few dermal neurofibroma were present. In addition, optic glioma was diagnosed.

Patient D, 38 years of age and mother of patient C, had mild learning difficulties during childhood, and showed macrocephaly and coarse facial features. As a child she showed signs of bony overgrowth similar to those seen in her son. In adult life these symptoms became less pronounced. Besides *café-au-lait* spots, freckling and Lisch nodules, an extreme number of dermal neurofibromas was observed.

Patient E, a woman of 33 years of age had mild to severe learning difficulties, attended normal schools but required remedial teaching. She lives with her parents as she is unable to live independently. A ptosis of the right eye was corrected, and coarse facial features with a slight micrognathia were observed. Besides multiple *café-au-lait* spots and an extreme number of dermal neurofibromas, also in the face, several plexiform neurofibromas located in the thorax, cervical- and lumbal spinal column, in the pelvic region and in the subcutaneous fat of the upper legs were detected. Complaints of ischiadic nerve compression could not be objectivated by additional investigations.

In summary, all patients showed intellectual impairment with varying severity (table 7.1). A variety of dysmorphic features were observed in all patients; at least 10 features of NF1 were recorded for each patient. We did not find an excessive number of dermal neurofibromas inappropriate for age, though two of the adult patients did have more than one hundred dermal neurofibromas (table 7.1).

Discussion

By screening DNA of 84 unrelated NF1-patients, not selected for dysmorphic features and intellectual impairment, we detected deletions encompassing the NF1 gene including the three genes embedded in intron 27, in 5 patients (from 4 unrelated families). The outer limits of three of the four deletions were defined by marker D17S117 (VAW212R2i) and marker D17S115 (VAW210M1). In the fourth deletion the centromeric boundary was also between D17S117 and GE2 but the telomeric end of the deletion was defined by marker D17S73 (EW207). The phenotype of these 5/84 patients resembled that of the 6 patients reported by Kayes et al. (4) as dysmorphic features and intellectual impairment were present in all patients. We are currently scrutinizing the clinical data on the non deletion patients to establish the overall frequency of severe intellectual impairment in our patients' group. However, as the frequency of moderate to severe intellectual impairment among NF1-patients has been estimated at 3.2% (14) the 5 patients with a deletion may well represent the majority of the patients with moderate to severe intellectual impairment.

Table 7.1
Phenotype of 5 patients with deletions encompassing NF1 gene

patient	A	B	C	D	E
age at latest examination (yr)	13	11	7	38	33
sex	f	f	m	f	f
family history NF1	-	-	+	+	-
parental origin deleted NF1 gene	M	F	u	u	u
≥ 6 café au lait spots	+	+	+	+	+
freckling	+	+	+	+	+
≥ 2 neurofibromas	+	-	+	+ +	+ +
≥ 1 plexiform neurofibroma	-	-	-	u	+ +
optic glioma	-	-	+	-	-
skeletal dysplasia	-	-	-	u	u
Lisch nodules	+	+	-	+	+
intellectual impairment	+	+ +	+ +	+	+
dysmorphic features	+	+	+	+	+
ptosis	+	-	u	u	+
deep-set eyes	-	+	u	u	-
epicanthal folds	-	-	+	u	-
prominent philtrum	+	+	+	u	+
micro/retrognathia	+	+	u	u	+
asymmetrical facial features	+	+	u	u	-
large ears	u	-	+	u	+
coarse face	+	+	u	+	+
pectus excavatum	+	-	u	u	u
cubitus valgus	+	-	u	u	u
abnormalities hands/feet	+	-	u	u	u
broad nasal bridge	+	+	u	u	+
overgrowth syndrome	-	-	+	+	-

f = female; m = male; F = father; M = mother; + = present; - = absent; u = unknown

Recently, Kayes et al. (4) reported deletions, spanning the entire NF1 gene in 5 out of 6 patients selected on the basis of dysmorphic features and learning disabilities. In 4 patients deletions were defined by D17S117 at the centromeric end of the NF1 gene, and by D17S115 at the telomeric end of the gene. In the fifth patient the outer boundaries of the deletion were defined by CRYB1 at the centromeric end of the NF1 gene and by D17S54 at the telomeric end of the gene (figure 7.4). The probes pAH1 and pAN2 were included in all deletions. In our subset we were not able to test these probes. As a consequence we are not certain if these probes are included in the deletions or not.

Figure 7.4
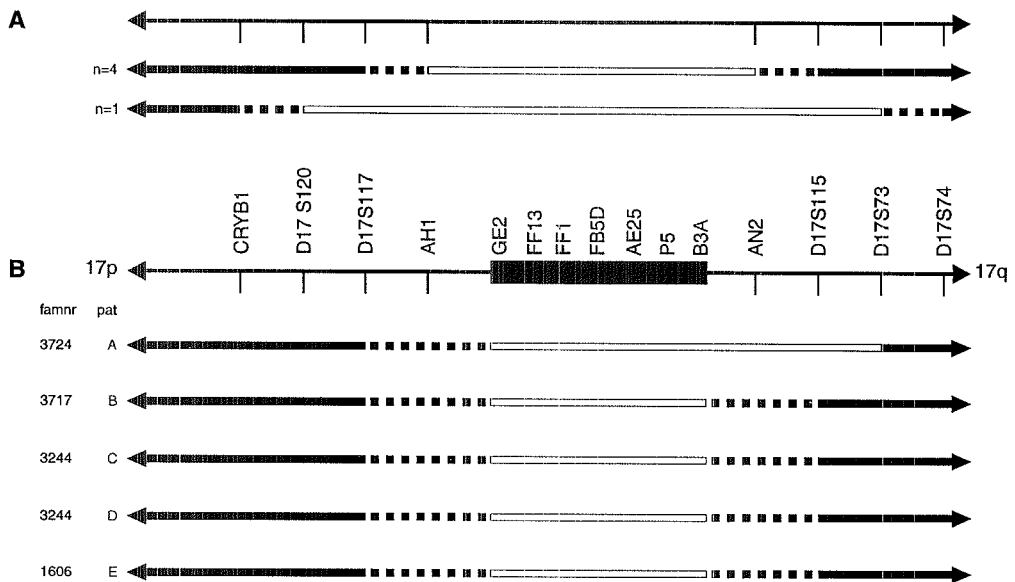*Extent of deletions in five patients from the present study (B) in comparison to the deletions as reported by Kayes (A). Thin line represents the region surrounding the NF1 gene, whereas the thick line represents the NF1 gene. Indicated are the NF1 cDNA probes and extragenic probes. Open bar: deleted region; dotted line: possibly deleted region; bar: region not involved in deletion. Not drawn to scale.*

In Kayes' study (4), an excessive number of dermal neurofibromas was observed in three post-pubertal patients. In addition, one prepubertal and one pubertal patient showed multiple neurofibromas and patches of pebbly textured skin, suggesting large numbers of subcutaneous neurofibromas. An excessive number of dermal neurofibromas was present in only two of our patients (patient D and E, both over 30 years of age). The presence of a gene in the vicinity of the NF1 gene, possibly involved in the development of dermal neurofibromas, as suggested by Kayes et al. (4), is not confirmed by our clinical findings as multiple dermal neurofibromas were only found in 2 of our 5 patients. However, it may be possible that such a gene is situated in the regions not included in our deletions.

In our study the patient with the largest deletion on the 3' end of the gene, had a greater number of dysmorphic features than the other patients, suggesting a possible association. However, the patient with a deletion extending slightly further to both the centromeric and the telomeric side in Kayes' subset did not show a disproportionate amount of dysmorphic features compared to the other patients. The degree of intellectual impairment in both our patient and Kayes' patient with a more extensive deletion, did not differ markedly from the other cases, excluding a gene responsible for mental development in the regions flanking the centromeric and telomeric end of the gene.

Until recently, mutational events in the NF1 gene on the maternally derived chromosome were considered quite rare (15). Soltan et al. (16) found hemizygosity in 9 patients after screening of 132 NF1-affected individuals with polymorphic markers; 6 were sporadic patients; in 4 of these 6 patients a deletion of the entire NF1 gene was present. Interestingly, all 4 patients showed a deletion on the maternally derived chromosome. It was suggested that the mechanism of gene-deletion may be more common on the maternally derived chromosome, in contrast to other types of mutations that may preferentially occur on the paternally derived chromosome. One of the four deletions detected in our study was of maternal origin, one of paternal origin and in one patient the origin of the deletion was unknown as the parents of the affected mother (patient D) were not available for DNA-analysis. As a consequence we have not been able to find evidence in support of this hypothesis.

The extreme variability in expression of some diseases makes genotype-phenotype studies of crucial importance for patients and their families. Precise predictions regarding genotype and phenotype in NF1 and other autosomal dominant diseases, such as tuberous sclerosis (17) and Marfan syndrome (18) have not been possible. Partly, due to mutational heterogeneity among families but also due to the intriguing clinical variability seen within families. In NF1 this has

presumably resulted in a low uptake of prenatal diagnosis (19). Our experience shows that even when prenatal diagnosis is requested parents seldomly decide to terminate the pregnancy.

An association between genotype and phenotype as reported here and by Kayes (4) may be the onset of an understanding of the influence of specific mutations in the NF1 gene on clinical features. Intrafamilial differences, such as seen in patient C and his mother, patient D, are presumably due to differences in genetic background. Easton et al. (20) have suggested that modifying genes play a key role in the variability of NF1 within families, showing highest correlation of number of *café-au-lait* spots and neurofibromas in monozygous twins. This correlation decreased as the genetic relationship became more distant.

Our results and those of a previous study, may imply that genotype-phenotype correlations may be present in NF1, in those cases showing deletions encompassing the entire NF1 gene. Future studies of multigenerational families are essential to further corroborate such a relationship.

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Chapter 8

Mutational and phenotypical analyses
in Neurofibromatosis type 1 patients

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Abstract

Neurofibromatosis type 1 (NF1) is one of the most common autosomal dominant disorders and is characterized by café-au-lait spots, skin fold freckling, neurofibromas and iris hamartomas (Lisch nodules). After mutation analysis in 200 unrelated NF1-patients, we detected mutations in 19 probands and a possible mutation in 1 proband.

Screening for large deletions resulted in the detection of 8 large deletions, 6 of which were shown to comprise the entire gene. With one exception, these cases showed a severe phenotype of mental retardation, dysmorphic features. The deletion was of maternal origin in 5 cases and of paternal origin in 1 case, in 2 cases it was undetermined. This series of entire gene/ large deletions brings the total number to 27 reported unrelated cases.

Small mutations were detected in 11 and possibly 12/192 (6%) NF1-patients after screening of approximately 12% of the NF1 coding region (exons 2, 24, 26, 28 and part of 31). Besides 6 novel mutations and one possible mutation, the recurrent mutations R1947X and K1423E were detected in 4 families (11 affected individuals) and 1 sporadic case, respectively. Phenotypes of probands and affected family members are discussed and compared to other reports.

Introduction

Type 1 Neurofibromatosis (NF1), formerly known as von Recklinghausen Neurofibromatosis, is an autosomal dominant disease with a prevalence of 1:3300 (1). Approximately one third of NF1-patients has 1 or more severe complications of the disease (2). As half of all patients represent de novo cases, the mutation rate of the NF1 gene (1×10^{-4} per gamete per generation) is one of the highest among human disease genes (3).

The NF1 gene was identified in 1990 and maps to chromosome 17q11.2 (4-6). It spans over 350 kb and contains approximately 60 exons (7). Three genes: *EVI2A*, *EVI2B* and the gene encoding oligodendrocyte-myelin glycoprotein (*OMGP*), have been detected within intron 27 (8, 9). *EVI2A* and *EVI2B* are human homologues of murine genes postulated to cause murine leukemia after activation by retroviral insertion. The *OMGP* gene encodes a protein expressed in myelinating Schwann cells and oligodendrocytes.

Neurofibromin, the protein encoded by the NF1 gene, consists of 2,818 amino acids with a predicted molecular mass of 327 kDa (10). A 360 amino acid region of the predicted protein product shows homology to the GTPase activating (GAP) family of proteins in yeast and mammals (11). The GAP-related domain (NF1-GRD) of neurofibromin is the only known functional domain of NF1 and spans exons 20 to 27a (bases 3497 - 4661) (12). The NF1-GRD of neurofibromin interacts with the product of the *Ras* protooncogene and this interaction may explain the tumor suppressor function. Neurofibromin is associated with cytoplasmic microtubules through parts of the gene product within the NF1-GRD (13). Hence, neurofibromin is considered to be involved in microtubule-mediated intracellular signal transduction (13).

The NF1 gene functions as a tumor suppressor gene in benign and malignant tumors associated with NF1 (14-17). In addition, somatic mutations in the NF1 gene have been observed in tumors unrelated to the disease (18-20).

Here, we report large deletions in 8 probands and small mutations and one possible mutation in 12 unrelated patients. Phenotypes are discussed in probands and affected family members.

Patients and Methods

This study included 200 unrelated patients, referred for DNA-studies and diagnosed according the criteria of the NIH. DNA was isolated from peripheral blood according to standard procedures (21). Patient DNA was screened for large deletions by microsatellite analysis and for small mutations by single strand conformation polymorphism (SSCP) analysis. Subsequently, large deletions were delineated by Southern blotting analysis and smaller mutations were identified by sequence analysis and confirmed by allele specific oligonucleotide hybridization (ASO). In all families DNA of available family members was tested.

Microsatellite analysis
Microsatellite analysis was performed by polymerase chain reaction amplification of the following polymorphic NF1 intragenic repeat markers: compound repeat in intron 26 (amplified fragment: 235-277 bp) (22), Alu repeat in intron 27 (395-407 bp) (23), CA repeat IVS27TG24.8 in intron 27 (270-292 bp), CA repeat IVS27AC28.4 in intron 27 (207-219 bp) (24) and CA repeat IVS38TG53.0 in intron 38 (171-187 bp) (25). Fragments were separated on a 6% polyacrylamide gel. If a monoallelic pattern, indicative for hemi- or homozygosity was observed,

Southern blotting analysis using cDNA clones of the NF1 gene was performed (with thanks to F.S. Collins).

Southern blotting analysis

For Southern blotting analysis 6 microgram of genomic DNA was digested with the restriction enzymes *Bam*H1, *Hind*III, *Bgl*II, *Eco*RI, and *Taq*I, according to the manufacturer's instructions. Fragments were separated on a 0.7% agarose gel. After blotting on Hybond N$^+$ membrane filters, the filters were hybridized with the overlapping cDNA probes GE2, FF13, AE25, P5 and B3A. Hybridization and autoradiography were performed according to standard procedures.

SSCP analysis

SSCP analysis was performed following Orita et al. (26). Sequences of primers used for amplification of exons 2, 24, 26, 28, and part of exon 31, and lengths of PCR products are depicted in table 8.1. PCR conditions for 15 microliters volume were 2.5 mM MgCl$_2$, 0.5 mM spermidine, 25 pmol of each primer, 200 mM mix of dATP, aGTP, dTTp, dCTP, and 2U of *Taq* polymerase. Thermal cycling conditions were 5 minutes at 94 degrees Celsius, followed by 30 cycles of 30 seconds at 94 degrees Celsius, 30 seconds at 55 degrees Celsius, 90 seconds at 72 degrees Celsius, with a final elongation of 10 minutes at 72 degrees Celsius. Two microliters of the PCR product were applied to the Pharmacia GenePhor Electrophoresis system. Gels were run for 2.5 hours at both 4 degrees Celsius and 15 degrees Celsius. Running conditions for 2 gels were 600 Volt, 50 mA and 10 Watt. Subsequently, the gel was colored using a DNA silver staining kit (Pharmacia) in a Hoefer automated gel stainer.

Sequence analysis

Amplifications of exons were performed in a 100 microliter volume using primers with an M13 tail. Subsequently, sequence analysis was performed with the cyclesequencing kit Dyeprimed (Perkin Elmer) on an ABI 377 automated sequencer.

ASO hybridization

ASO hybridizations were performed at 37 degrees Celsius for 30 minutes. Filters were washed to 0.3 SSC for 10 minutes at 37 degrees Celsius. Sequences of oligonucleotides for ASO hybridization will be communicated on request.

Table 8.1
Primer sequences for exon amplification in SSCP analysis for NF1 mutation detection

Exon	Primers	Fragment length (bp)
2	5' TTAAGGATAAACTGTTTACGTG 3' 5' ACACAGTAACCCAAATACTCAC 3'	211
24	5' CATGTCTTTATATTAATTCAAACCT 3' 5' ATGTAAGAGGAAGTACTGTAAG 3'	296
26	5' TGAAAATTCTAATGACTTTG 3' 5' CATGACCAATAATTGTATTA 3'	251
28	5' CACTGCTAATAATCTTTGTCTTTTTTGTC 3' 5' ATCCGTTTACAAAACACAGACTGGAACTTA 3'	504
31	5' ATAATTGTTGATGTGATTTTCATTG 3' 5' CCATATTTTTGCTTGAATAGA 3'	240 [1]

[1]   SSCP analysis was performed on a fragment of exon 31.

Results

Mutation analysis

After screening for large deletions and mutation analysis of exons 2, 24, 26, 28, and part of exon 31, which comprises approximately 12% of the coding region (excluding promoter region), 19 mutations and 1 possible mutation were found in 20 probands (34 affected individuals) from a series of 200 unrelated NF1-patients. Mutations detected in this study are summarized in table 8.2.

In 6 families, deletions were found encompassing the entire NF1 gene, including the embedded genes in intron 27 (*EVI2A, EVI2B, OMGP*). Also, two large deletions were detected: one deletion encompasses at least exon 9 to 50 (family 6387). The second deletion involves at least exons 27 through 38 (family 9634). One of the in total 8 large deletions, was inherited (family 3244).

The results of microsatellite analysis in families 1606, 3717, 3244 and 3724 were reported earlier (27). Family 5589 was informative for the polymorphic markers IVS27TG24.8 and IVS38TG53.0. Family 5635 showed hemizygosity for the polymorphic compound repeat in

Table 8.2

Twenty mutations detected in 200 unrelated patients with NF1

Family number	Family history	Affected individuals	Parental origin	Exon	DNA sequence change	Expected effect at protein level
Mutations						
1606*	-	1	n.i.	1 - 60	entire gene deletion	-
3244*	+	2	n.i.	1 - 60	entire gene deletion	-
3717*	-	1	P	1 - 60	entire gene deletion	-
3724*	-	1	M	1 - 60	entire gene deletion	-
5589	-	1	M	1 - 60	entire gene deletion	-
5635	-	1	M	1 - 60	entire gene deletion	-
6387	-	1	M	9 - 50	large deletion	-
9634	-	1	M	27 - 38	large deletion	?
7361	-	1	n.i.	2	81 GC->AT	Q28X
8867	+	3	n.i.	24	4111-2 A->G	aberrant splicing
8439	-	1	n.i.	24	4267 A->G	K1423E
9271	-	1	n.i.	24	4269 G->T	K1423N
7860	+	5	n.i.	26	4368-1 G->A	aberrant splicing
7415	-	1	n.i.	26	4514+1 G->T	aberrant splicing
4740	-	1	n.i.	28	4805del7	truncation
2039	+	5	n.i.	31	5839 C->T	R1947X
5206	-	1	n.i.	31	5839 C->T	R1947X
8456	+	2	n.i.	31	5839 C->T	R1947X
9851	+	3	n.i.	31	5839 C->T	R1947X
Mutation/ Polymorphism						
1575	+	2	n.i.	intron 26	4514+12 C->T	?

+ = present; - = absent; nt = not informative; n.i. = not informative; P = paternal; M = maternal; * reported earlier (27)

intron 26 and the Alu repeat in intron 27 (figure 8.1A). Family 6387 lacked a maternal contribution when tested with the marker IVS27AC28.4 and family 9634 when markers IVS27AC28.4 and IVS38TG53.0 were tested.

The results of Southern blotting analysis in families 1606, 3717, 3244 and 3724 were reported elsewhere (27). DNA of the probands of families 5589 and 5635 showed reduced signals on Southern blotting analysis for the overlapping cDNA probes GE2, FF13, AE25, P5 and B3A (figure 8.1B). It was concluded that their deletions were at least 350 kb in length; the extragenic boundaries are still to be established. The exact extent of the large deletions in probands 6387 and 9634 is undetermined: in proband 6387 decreased intensities of the hybridizing fragments were observed for cDNA probes FF1, AE25 and P5, as compared to his unaffected parents. In proband 9634, Southern blotting analysis is still to be performed.
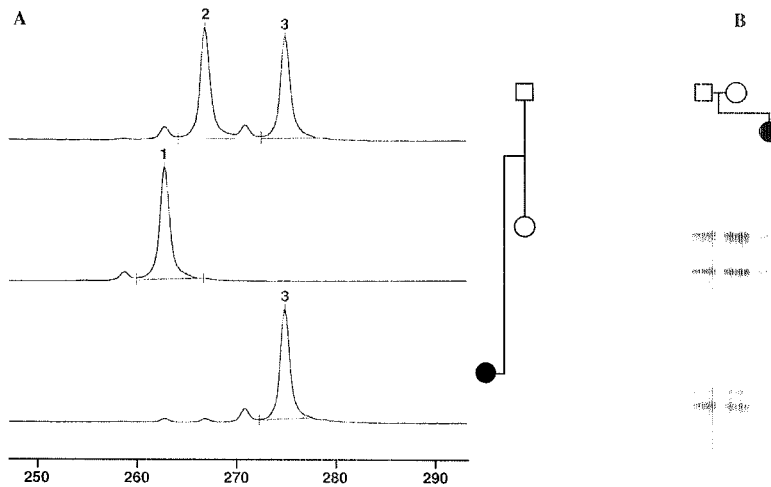
Figure 8.1
A) The intragenic compound repeat in intron 26 shows hemizygosity for the paternal allele in the affected child (patient 5635). B) Southern blotting analysis using MspI digested genomic DNA and intragenic cDNA probe P5 shows a decreased intensity in the DNA of the affected child (patient 5635) as compared to the DNA of her unaffected parents. DNA concentrations are equalized.

In 5 of these 8 families the deletion was of maternal and in 1 of paternal origin, in 2 cases undetermined due to either lack of parental DNA or marker informativeness.

Nonsense mutations were detected in 5 families, one of these resulted in a stopcodon at amino acid position 27 in exon 2 (81 GC->AT). In the other 4 families, the recurrent

mutation R1947X in exon 31 (5839 C->T) was detected, with multiple cases in one family (2039) (table 8.2).

In 3 families, splice site mutations were detected in respectively, exon 24 (4111-2 A->G), and exon 26 (4368-1 G->A; 4514+1 G->T). Families 8867 and 7860 showed transmission of these mutations. In exon 24, 2 missense mutations were found: the recurrent missense mutation K1423E (4267 A->G) and a novel missense mutation K1423N (4269 G->T). In both families, the parents were clinically unaffected and tested negative for the mutation. In addition, K1423N was not detected in 100 healthy controls. In 1 family a small deletion of 7 base pairs was detected in exon 28, starting at base 4805 (4805del7).

Furthermore, in 1 family a possible mutation 4514+12 C->T (intron 26) was detected in 2 affected family members and undetected in 3 unaffected relatives.

When possible, probands and their family members were tested by ASO hybridization. Aims were to confirm the association of the mutation with the disease, to identify patients with an *NF1* gene mutation but insufficient diagnostic criteria and to exclude possible somatic mosiacism in the leucocytes of the parents. Figure 8.2 shows the results of ASO hybridizations in a number of families with a small mutation (or possible mutation).

Clinical data
Clinical manifestations of all probands and affected family members are summarized in tables 8.3, 8.4 and 8.5.

Clinical manifestations in families with large deletions
Clinical data of the 8 unrelated patients with large deletions are summarized in table 8.3 and include 4 probands (1606, 3244, 3717 and 3724) reported previously (27). One proband (3244) had an NF1-affected mother. Her clinical data are also included. Eight of the in total 9 individuals with a large deletion, presented with a combination of intellectual impairment and dysmorphic features. One patient with multiple dermal neurofibromas at a young age (proband 5635) had neither intellectual impairment nor dysmorphisms. Dysmorphic features (excluding macrocephaly and hypertelorism), in the 8 patients with a similar severe phenotype included: full lips, a prominent mandible and maxilla and large ears (family 5589). Also, a coarse face, epicanthal folds, large, coarse hands with a simian crease and clinodact-yly of the fifth finger were observed (the proband in family 6387). The proband in family 9634 was initially diagnosed with Noonan syndrome. Subsequently, *café-au-lait* spots and skin fold freckling were noticed. He shows antimongoloid slanting of the eyes, asymmetrical orbitae, micrognathia and a high palate.
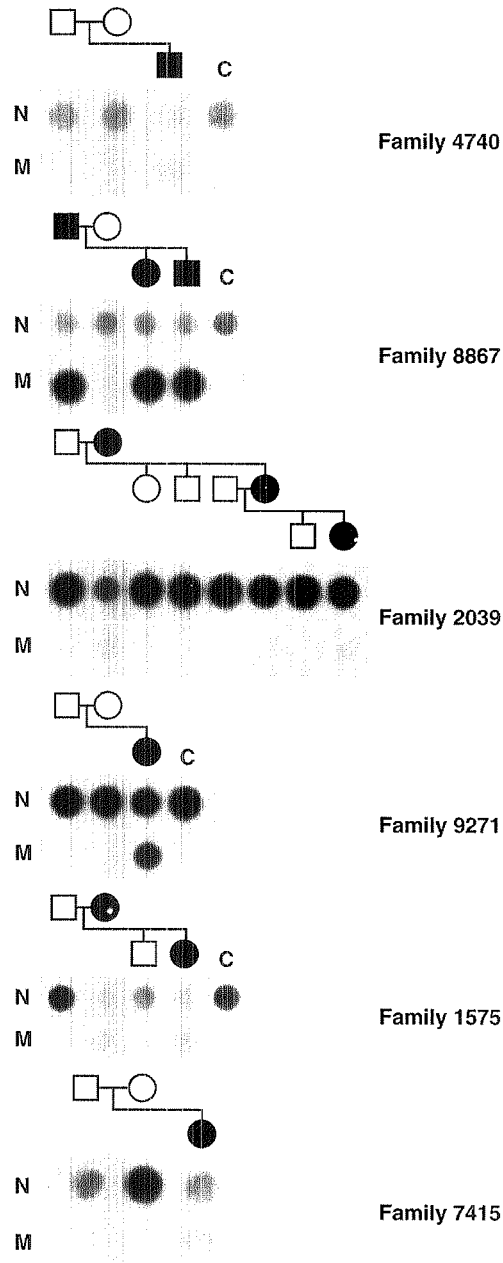
Figure 8.2

ASO hybridizations of families 4740, 8867, 2039, 9271, 1575 and 7415 (affected family members are shaded) with oligonucleotide with normal sequence (N) and mutated sequence (M).

Table 8.3

Phenotype of large NF1 gene deletions in 8 families (9 individuals)

Family number	1606	3244		3717	3724	5589	5635	6387	9634
Affected relatives (R)		R1	R2						
General features									
Age at last examination	33	7	38	11	13	34	33	20	3
Sex	f	m	f	f	f	f	f	m	m
Intellectual impairment	+	++	+	++	+	++	-	++	++
Dysmorphic features	+	+	+	+	+	++	-	+	++
Acromegalic features	-	-	-	-	-	-	-	+	-
Major disease features									
≥6 *café-au-lait* spots	+	+	+	+	+	+	+	+	+
Freckling	++	+	+	+	+	+	+	+	+
≥ 2 dermal neurofibromas	++	+	++	-	+	+	++	+	-
≥ 1 plexiform neurofibroma	++	-	u	-	-	-	-	+	-
Lisch nodules	+	-	+	+	+	-	+	+	u
Optic glioma	-	+	-	-	-	-	-	-	u
Osseous lesions specific for NF1 (sphenoid dysplasia, bowing of long bones)	u	-	u	-	-	-	-	-	-
Complications observed									
Valvular pulmonary stenosis	-	-	-	-	-	-	-	-	+
Scoliosis	-	-	-	-	-	-	-	+	-
Strabismus	-	-	-	-	-	-	-	+	-
Overgrowth syndrome	-	+	+	-	-	-	-	-	-

Family numbers in bold from: (27); + = present; - = absent; u = unknown; f = female; m = male

Clinical manifestations in families with recurrent mutations

Clinical data of 4 unrelated families with the recurrent nonsense mutation R1947X (5839 C->T) with a total of 11 affected individuals are presented in table 8.4.

Patient R1 from family 2039 is a girl with familial NF1 diagnosed at 7 years of age, with *café-au-lait* spots and freckling and a plexiform neurofibroma on the right upper leg and above the left ankle. She experienced mild learning-, speech- and motor difficulties. She developed Lisch nodules in both irides and dermal neurofibromas on the trunk. Her mother (R2), 2 aunts (R3,R4) and grandmother (R5) were also diagnosed with the disorder. The mother (R2) showed *café-au-lait* spots, dermal neurofibromas and Lisch nodules. The dermal (subcutaneous) neurofibromas appeared during her first pregnancy and are painful. One aunt with NF1 (R3) (*café-au-lait* spots, mental retardation, scoliosis) died at 9 years of age due to a primary brain tumor, with intraspinal extensions. The second aunt (R4) presented with *café-au-lait* spots, dermal neurofibromas and Lisch nodules. The grandmother (R5) shows *café-au-lait* spots, Lisch nodules, excessive, often painful dermal neurofibromas and has been operated for a diffuse plexiform neurofibroma of left leg and foot, and for a pyelo-urethral stenosis.

In family 5206, the proband was diagnosed as a de novo case at 9 years of age with *café-au-lait* spots and freckling. At 15 years of age, he has developed dermal neurofibromas but is complication-free.

In family 8456, the proband R1 has *café-au-lait* spots, freckling, dermal neurofibromas, Lisch nodules, a symptomatic non progressive optic pathway glioma and mental retardation. He shows downslanting eyes, epicanthal folds, low set ears, a webbed neck and a high palate. His mother (R2) was found affected with *café-au-lait* spots, freckling, dermal neurofibromas and epileptic seizures.

Family 9851 also shows multigenerational NF1. The proband (R1) shows *café-au-lait* spots, freckling, dermal neurofibromas. A left tibia recurvata with a persistent pseudoarthrosis has been amputated. Moreover, he has presented with a retinoblastoma of the left eye and Crohn's disease. Two brothers (R2; R3), who have not been examined by a physician, are reported with areas of hyperpigmentation and tumors. The parents have died, the mother of uterine cancer and the father of a lung carcinoma.

In family 8439, the recurrent mutation K1423E (4267 A->G) was detected. The proband was a de novo case and presented with macrocephaly, hypertelorism, *café-au-lait* spots, and few dermal neurofibromas (table 8.5).

Table 8.4

Phenotype of R1947X mutation (exon 31) in 4 families (11 individuals)

Family number	2039					5206	8456		9851		
Affected relatives (R)	R1	R2	R3	R4	R5		R1	R2	R1	R2*	R3*
General features											
Age at last examination	17	36	9	30	69	15	12	30	43	49	51
Sex	f	f	f	f	f	m	m	f	m	m	m
Intellectual impairment	-	-	+	-	-	-	+	-	+	u	u
Dysmorphic features	-	-	-	-	-	-	+	-	-	-	-
Acromegalic features	-	-	-	-	-	-	-	-	-	-	-
Major disease features											
≥6 *café-au-lait* spots	+	+	+	+	+	+	+	+	+	+	+
Freckling	+	u	u	+	+	+	+	+	+	u	u
≥ 2 dermal neurofibromas	+	+	-	+	++	+	+	+	+	+	+
≥ 1 plexiform neurofibroma	+	-	-	-	++	-	-	-	-	-	u
Lisch nodules	+	+	u	+	+	-	+	-	u	u	u
Optic glioma	-	-	u	-	-	-	+	-	-	u	u
Osseous lesions specific for NF1 (sphenoid dysplasia, bowing of long bones)	-	-	-	-	-	-	-	-	+	-	-
Complications observed											
Primary brain tumor	-	-	+	-	-	-	-	-	-	-	-
Scoliosis	-	-	+	-	-	-	-	-	-	-	-
Epilepsy	-	-	-	-	-	-	-	+	-	-	-
Retinoblastoma	-	-	-	-	-	-	-	-	+	u	u
Crohn's disease	-	-	-	-	-	-	-	-	+	-	-

+ = present; - = absent; u = unknown; f = female; m = male; * = no physical examination

Clinical manifestations in families with novel mutations

In proband 7361, a nonsense mutation was detected in exon 2 (Q28X). Clinical manifestations included *café-au-lait* spots, dermal neurofibromas and a plexiform neurofibroma.

In family 8867, a splice site mutation was found in exon 24 (4111-2 A->G). The proband (R1) presented with only *café-au-lait* spots. Furthermore, hypertelorism, epicanthal folds, an abnormal anterior thorax curvature and mental retardation were observed. At 6 months of age, he developed a juvenile chronic myeloid leukemia (JCML). In search of a bone marrow donor, *café-au-lait* spots were observed in a sibling (R2) and a few neurofibromas were found in the father (R3). Firstly, linkage analysis showed that both the proband (R1) and the sibling (R2) had the same paternal intragenic haplotypes of the NF1 region. Subsequently, NF1 was diagnosed in the three family members (R1, R2, R3) by mutation detection. As a consequence the sibling and the father were excluded as potential bone marrow donors.

In family 9271, the missense mutation K1423N was detected in exon 24 (4269 G->T). Atypically, the proband developed *café-au-lait* spots only from 3 years of age, subsequently followed by freckling at 5 years. From early infancy, she developed hypsarrithmia because of an arachnoid cyst in the left temporal region. She is severely mentally retarded and has a right hemiparesis.

A splice site mutation in exon 26 (4368-1 G->A) was found in proband 7860 (R1) and in her affected father (R2) and brother (R3). In addition, an uncle and grandfather are reported to have symptoms of the disease (no clinical data and DNA available). The proband shows *café-au-lait* spots, axillary and inguinal freckling, one subcutaneous neurofibroma, Lisch nodules and a mild scoliosis. In the father, a neurofibroma of the occipital nerve was extirpated. Furthermore he shows *café-au-lait* spots, hundreds of dermal neurofibromas, pseudotrophic maculae, Lisch nodules and also a mild scoliosis. Her affected brother shows *café-au-lait* spots, skin fold freckling, a dermal neurofibroma, is macrocephalic and has a short stature.

Another splice site mutation was detected in exon 26 (4514+1 G->T) in family 7415. The proband presented with *café-au-lait* spots, skin fold freckling, a progressive optic chiasma glioma responding to radiotherapy, and a characteristic hyperintense region in the mesencephalon on T2-weighted MRI images (28). Subsequent MRI-imaging showed diffuse brainstem enlargement, with mass effect and gadolinium enhancement in the area originally regarded as a hyperintense region. No neurological symptoms were observed. Six months later she presented with headaches, nausea and left hemiparesis; chemotherapy was initiated.

Table 8.5 (A)

Phenotypes of 8 mutations in exons 2, 24, 26 and 28 in 8 families (14 individuals)

Exon	exon 2	exon 24				
Mutation	Q28X	4111-2 A->G			K1423E	K1423N
Family number	7361	8867			8439	9271
Affected relatives (R)		R1	R2	R3		
Age at last examination	33	4	7	37	34	6
Sex	m	m	f	m	m	f
Intellectual impairment	-	+	+*	-	-	++
Dysmorphic features	-	+	-	-	-	+
Acromegalic features	-	-	-	-	-	-
Major disease features						
≥6 *café-au-lait* spots	+	+	+	-	+	+
Freckling	+	-	-	-	u	+
≥ 2 dermal neurofibromas	+	-	-	+	+	-
≥ 1 plexiform neurofibroma	-	-	-	-	-	-
Lisch nodules	+	-	-	-	-	-
Optic glioma	-	-	-	-	-	-
Osseous lesions specific for NF1 (sphenoid dysplasia, bowing of long bones)	-	-	-	-	-	-
Complications observed						
Juvenile chronic myeloid leukemia	-	+	-	-	-	-
Astrocytoma	-	-	-	-	-	-
Hypsarrithmia	-	-	-	-	-	+

+ = present; - = absent; u = unknown; f = female; m = male; * = mental retardation due to severe perinatal asphyxia

Table 8.5 (B)

Phenotypes of 8 mutations in exons 2, 24, 26 and 28 in 8 families (14 individuals)

Exon				exon 26			exon 28
Mutation	4368-1 G->A			4514+1 G->T	4514+12 C->T		4805del7
Family number	7860			7415	1575		4740
Affected relatives (R)	R1	R2	R3		R1	R2	
Age at last examination	15	49	11	11	19	44	20
Sex	f	m	m	f	f	f	m
Intellectual impairment	-	-	-	+	-	-	++
Dysmorphic features	-	-	-	+	-	-	-
Acromegalic features	-	-	-	-	-	-	-
Major disease features							
≥6 *café-au-lait* spots	+	+	+	+	+	u	+
Freckling	+	+	+	+	+	u	u
≥ 2 dermal neurofibromas	-	+	-	+	-	u	+
≥ 1 plexiform neurofibroma	-	-	-	-	-	u	+
Lisch nodules	+	+	-	+	u	u	u
Optic glioma	-	-	-	+	u	u	u
Osseous lesions specific for NF1 (sphenoid dysplasia, bowing of long bones)	-	-	-	-	-	u	-
Complications observed							
Juvenile chronic myeloid leukemia	-	-	-	-	-	-	-
Astrocytoma	-	-	-	+	-	-	-
Hypsarrhythmia	-	-	-	-	-	-	-

+ = present; - = absent; u = unknown; f = female; m = male; " = mental retardation due to severe perinatal asphyxia

In family 1575, a possible mutation was detected in intron 26 (4514+12 C->T) in the proband and affected mother. The proband (R1) showed *café-au-lait* spots and freckling at 5 years of age. Her mother (R2) was also diagnosed with NF1 by a clinical geneticist. No information on her phenotype is available.

A small deletion was detected in exon 28, in proband 4740. This young man showed intellectual impairment, *café-au-lait* spots, dermal and plexiform neurofibromas.

Discussion

Mutation detection
In this study of 200 NF1-patients, 6 and possibly 8 entire gene deletions were found (4 were reported earlier) (27). This study brings the total of reported entire gene/ large deletions to 27. Furthermore, small mutations were detected in 11 and possibly 12/192 NF1-patients (6%) after screening of approximately 12% of the coding region. These mutations do include 4 cases of the recurrent mutation R1947X in exon 31, which were efficiently detected by ASO hybridization. The exons 24, 26 and 28 were selected for mutation analysis as they are situated within the NF1-GRD, the only region to which a biological function has been ascribed. These exons yielded 7 different mutations, 6 of which were novel. Exon 2 was chosen as it had received little attention in programs for mutation analysis; one novel mutation was detected in this exon. Exon 31 was selected as it is the site of the recurrent mutation R1947X, but additional, different mutations were not identified.

Despite the development of new techniques based on both DNA and RNA analysis (29-33), studies report mutation detection in only 10-20% of clinical series (34). This detection rate is generally attributed to the large size of the gene, and the paucity of recurrent mutations. Somatic mosaicism as found in 3 patients with a generalized NF1, has also been suggested to be a complicating factor in mutation analysis (35-37). However, when the detection rate is related to the percentage of screened exons or coding region, results vary from 30% to almost 100% (30, 38, 39). Our results (50%) are in accordance to these recent mutation reports and suggest that mutation detection rates may not be as low as has been reported.

Although few in number, recurrent mutations have been found. Including our study, the non-sense mutation in exon 31 R1947X (5839 C->T), has been described in 15 unrelated NF1-patients (4, 29, 38, 40-43). The missense mutation K1423E (4267 A->G) has been reported in 5 unrelated patients (19, 44). Our study brings the total to 6. This mutation has also been reported as a somatic mutation in 3 unrelated tumors (19). Interestingly, we detected a novel mutation, K1423N, affecting the same codon. Furthermore, within a 4 base pair sequence

in exon 37, a total of 6 mutations have been found (30). Also, a small deletion in exon 17 (2970delAAT) and an insertion (5451insC) in exon 29 were each detected twice in 150 unrelated cases (45).

Large deletions and phenotype

Table 8.6 shows an overview of studies of NF1 patients with large deletions in 27 unrelated families with 3 affected relatives in 3 families (27, 37, 46-51). The most widely used detection methods have been quantitative Southern blotting analysis with intragenic and extragenic probes, analysis of locus hemizygosity using polymorphic markers (27, 51) and fluorescence *in situ* hybridization (FISH) (47-50). In 18 out of 27 unrelated cases, deletions were at least 350 kb; 16 of these were proven to be >700 kb in length, suggestive for a possible contiguous gene syndrome.

The initial five patients with an entire NF1 gene deletion presented with a phenotype of intellectual impairment, dysmorphic features and multiple neurofibromas at an early age (46). This rather severe phenotypical expression was confirmed in the majority of subsequently reported cases (27, 47-50).

In addition to mental retardation in 27/30 and dysmorphic features in 24/30 reported cases, excessive neurofibromas were observed in 16/30 cases. Plexiform neurofibromas, were reported in approximately half of the cases, whereas their overall occurrence in NF1 is approximately 30% (2, 52).

Recurrent mutations and phenotype

This study added 4 unrelated families with 11 affected individuals with the recurrent nonsense mutation R1947X, bringing the worldwide total to at least 14 probands (and 29 affected individuals). The mutability at this "hot spot" is presumably partly due to the presence of a methylated cytosine (56). DNA methylation of CpG dinucleotides is considered a major contributor of point mutations leading to human disease, since it may precede deamination of 5-methylcytosine (57). There is both a large intrafamilial and interfamilial variability in phenotypic expression in patients with this nonsense mutation. A predisposition to scoliosis has been suggested but was observed in only one of our cases (40).

The missense mutation K1423E has now been reported in 6 unrelated families. The effect of this mutation on GAP activity is important as shown by a 200 to 300-fold reduction of GAP activity in site-directed mutagenesis expression studies (19). Phenotypic information is only available in our patient (family 8439) who showed a mild phenotype, with only *café-au-lait* spots and few neurofibromas.

In family 9271, the missense mutation K1423N was detected in exon 24 and considered pathological as it was neither detected in the proband's unaffected parents nor in 100 healthy controls. This proband showed a late onset of *café-au-lait* spots (only from 3 years), atypical for the disease, severe mental impairment and a history of hypsarrithmia. Interestingly, probands from families 8439 and 9271 with mutations within the same codon (1423) show no phenotypical similarites.

Valero et al. (51) and our group observed normal intelligence and absence of dysmorphic features, in a total of 3/27 large deletion patients; however, the patient in our study had early appearing multiple dermal neurofibromas. In conclusion, entire gene deletion patients show a tendency towards a more severe phenotype. The clinical variability, although present, seems less extreme than in patients with smaller mutations.

The clinical variability of large deletions may be influenced by the extent or the origin of the deletion. Deletion of flanking genes might be associated with the more severe symptoms such as intellectual impairment, dysmorphic features, the early and severe development of dermal neurofibromas and plexiform neurofibromas. Until the boundaries of the deletions are delineated, no definite associations can be made. In our series, the parental origin of the deletion did not seem to influence the severity of the phenotype. The origin of large deletions was maternal in 12/27 cases, paternal in 4/27 cases, and unknown in 11/27 cases (table 8.6). Although it is likely that large deletions may occur more often in the maternal DNA due to an increased frequency of recombinational error during meiosis (53-55), there is no definite evidence for this.

Novel mutations and phenotype

The oligosymptomatic phenotypes of the indexpatient R1 in family 8867 (*café-au-lait* spots and JCML), his sibling (R2), and father (R3) all insufficient for a diagnosis of NF1, illustrates the extreme clinical variability observed in NF1-patients. On the other hand, this case underlines the diagnostic and therapeutic relevance of mutation analysis in the NF1 gene, excluding sister and father as potential bone marrow donors for R1.

In family 1575 the proband and her affected mother shared the mutation 4514+12 C->T in intron 26. It was not detected in 3 unaffected relatives. The exact effect at protein level is unclear as the transition seems too far into the intron to affect splicing. Further analysis of the family, both at DNA and RNA level, is necessary to establish the nature of this sequence variation.

Table 8.6 (A)

Overview of 30 reported cases of large deletions in the NF1 gene

		Kayes	Wu	Upadhyaya	Riva	Leppig	Valero	This report	Total
Unrelated families (n)		5	5	1	1	2	5	8	27
Affected relatives (n)		0	1	0	0	0	1	1	3
Parent of origin	maternal	1	-	1	1	0	4	5	12
	paternal	2	-	0	0	0	1	1	4
	unknown	2	5	0	0	2	0	2	11
Mental retardation or learning disabilities		5	6	1	1	2	4	8	27
Dysmorphic features		5	6	1	1	2	1	8	24
Excessive dermal neurofibromas		5	5	0	0	2	1	3	16
Other features	plexiform neurofibromas	5	2	0	0	0	5	2	14
	malignancies	0	1	0	0	0	3	0	4
	skeletal deformities	0	0	0	1	0	0	2	3
	epilepsy	0	1	1	0	0	0	0	2
	Madelung deformity	0	0	0	0	1	0	0	1
Extent deletion	7 Mb	0	0	1	0	0	0	0	1
	> 700 kb	5	5	0	1	0	0	4	16
	≥ 350 kb	0	0	0	0	0	0	2	2
	≥ 275 kb	0	0	0	0	2	0	0	2
	≥ 225 kb	0	0	0	0	0	5	1	6
	≥ 2.5 kb (exons 27 - 38)	0	0	0	0	0	0	1	1

+ = present; - = absent

Table 8.6 (B)

Overview of 30 reported cases of large deletions in the NF1 gene

	Kayes	Wu	Upadhyaya	Riva	Leppig	Valero	This report	Total
Unrelated families (n)	5	5	1	1	2	5	8	27
Affected relatives (n)	0	1	0	0	0	1	1	3
Technique								
somatic cell hybrids	+	-	-	-	-	-	-	1
Southern blot analysis	+	-	+	-	-	+	+	4
analysis of locus hemizygosity	-	-	+	+	-	+	+	4
gene dosage PCR-assay	-	-	+	-	-	-	-	1
high resolution G banding	-	-	+	-	-	-	-	2
FISH	-	+	+	+	+	-	-	4

+ = present; - = absent

Genotype-phenotype studies

In our series of 200 NF1-patients we report 19 and possibly 20 mutations in 34 affected individuals. Due to our selection of screened exons, most mutations affect the NF1-GRD, the only known region with a biological function. It may be concluded that mutations in this region show the same clinical variability observed in patients with mutations in other areas. Other functional domains within the NF1 gene are still to be identified. Also, our data illustrate the large intrafamilial and interfamilial variability in phenotypic expression between NF1-patients with identical or similar mutations. This phenotypic variability is a major contributor to the difficulties and anxiety NF1-families experience, when informed of the disease and its natural history or when confronted with the option of prenatal diagnosis.

The absence of straightforward relationships between genotype and phenotype in NF1 shows the complexity of this "monogenic" disease. Studies in other autosomal dominant diseases such as von Hippel-Lindau disease (58) and tuberous sclerosis show similar results. Presumably, other factors such as modifying genes (59) and environmental factors play a role in the developing phenotype. Future studies focusing on the clinical diversity within NF1 families, such as affected sib-pair analysis, may possibly uncover candidate genes involved in the expression of the phenotype and help elucidate the pathogenesis of this common genetic disease.

Acknowledgments

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Chapter 9

General discussion and future prospects

9.1   Clinical studies and NF1 pathogenesis

In 1974, Bolande classified NF1 as a neurocristopathy or a neural crest disorder: a disease due to abnormalities in embryological migration or differentiation of neural crest cells (1). Indeed, the main cell types involved in the major disease features in NF1 are of neural crest origin. For instance the melanocytes in *café-au-lait* spots and skin fold freckling, the Schwann cells in neurofibromas and the iris with its pigmented Lisch nodules. However, not all major disease features can be ascribed to a neural crest disorder. NF1 is a progressive disease and subsequent symptoms and complications occur in all organ systems, irrespective of their embryological origin. Little is known of the interactions during development and senescence, between neurofibromin, the neural crest and other structures. Clinical studies are indispensable for a further understanding of the pathogenesis of NF1, as careful observation and documentation of the natural history of the disease may uncover interactions and possible modifiers, and provide new insights on the clinical expression of the disease.

In our prospective 10 year follow-up study (chapter 2) we reported the prevalence of the major disease features and symptoms and complications related to NF1 in a large group of children. Moreover, we were the first to report an incidence rate of complications in NF1-patients. Documentation of the occurrence of complications during follow-up and analysis of risk factors associated with the development of complications is a step towards unraveling the complex pathogenesis of NF1.

Studies such as these in which longitudinal data are collected on the developing phenotype, are only possible in a multidisciplinary setting, where surveillance by experienced NF-specialists is possible. These clinics are able to provide optimal information and support for patients and their families. Patient management has benefited and will continue to benefit from the experiences acquired in these clinics as the following examples will illustrate.

Elucidation of the natural history of specific complications may have important therapeutic consequences. Extensive prospective MRI-imaging studies on the progression of optic pathway glioma in NF1-patients has changed clinical management of these generally benign tumors as the aggressive surgical approach was definitely abandoned for an attitude of expectation (2). In addition, our prospective 10 year follow-up study showed that visual evoked potentials are a valuable addition to ophthalmological examinations for the diagnosis ofoptic pathway glioma in children with NF1. This was confirmed by North et al. (3) Furthermore, clinical studies have shown that additional examinations (blood, urine, X-ray, ultrasound, CT-scan or MRI) for NF1-related complications should only be performed on medical indication (4, 5).

Similar prospective follow-up studies are necessary on the natural history of dermal- and plexiform neurofibromas, two frequently occurring major disease features. What are the effects of puberty, pregnancy and other hormonal factors, such as growth hormone therapy (for growth hormone deficiency), on these disfiguring, and in the case of the plexiform neurofibromas sometimes lethal tumors? What are the effects of surgery on plexiform neurofibromas and at what age are the results most pronounced? Moreover, until the natural course of NF1-specific complications is revealed, the results of future therapeutic trials may be difficult to interpret.

In the near future, the quest for modifying genes will be initiated. Affected sib-pair analysis may play a decisive role in this pursuit. This genetic epidemiological technique compares the clinical expression of the disease in affected siblings with the genomic haplotype of the affected siblings (favorably in regions containing candidate genes). In this way, candidate genes for modifying functions may be detected. Affected sib-pair analysis has been used to identify genetic factors implicated in complex diseases such as insulin-dependent diabetes mellitus and migraine (6-8). Multidisciplinary NF1 clinics, particularly clinics with a young population such as ours, will be important recruiters of participants for these studies.

9.2   Diagnostic problems

Although NF1 diagnosis may often be straightforward, exceptions are observed and form a major problem for the physician or genetic counselor involved. Oligosymptomatic manifestations and segmental distributions of major disease features may lead to atypical presentations requiring a divergent approach to make a timely diagnosis (as in family 8867, chapter 8). On the other hand, molecular genetic analysis of atypical cases may lead to insights in the extent of the disease spectrum and the pathogenesis of NF1.

In chapter 3, we have analyzed the diagnostic value of the minor disease features: macrocephaly, short stature, hypertelorism and thorax abnormalities, in children $\leq$ 6 years of age and suspected of NF1. It was concluded that in children with insufficient diagnostic criteria for NF1 and $\leq$ 6 years of age, documentation of minor disease features may be a helpful aid in predicting the diagnosis of NF1 in years to come. In the future, the minor disease features may also be of help in diagnosing atypical cases. However, definite conclusions on the applicability of these minor disease features can only be made when mutation analysis has become diagnostic in the majority of families.

Since the establishment of the diagnostic criteria (9), early diagnoses of NF1 became possible through the combination of a positive family history and the presence of one diagnostic criterion, generally *café-au-lait* spots. Recognition of the disease in early infancy may be beneficiary. Informing the parents on the disease and its complications becomes possible, securing that NF1-related complications are recognized. Also, the parents and relatives can be informed of the inheritance of the disease, can be screened themselves, and genetic counseling and the possibility of prenatal diagnosis may be offered.

In contrast, retrospective diagnosis in a parent due to diagnosis of NF1 in a child (first degree relative) may cause dilemmas in some cases. When NF1 is diagnosed on the basis of the (clinical) diagnostic criteria in the child, and sufficient (clinical) diagnostic criteria are found in one of the parents, NF1 diagnosis in the parent and the risk for future offspring is straightforward.

However, if NF1 in the parent is diagnosed on the basis of one (clinical) diagnostic criterion and an affected first degree relative, NF1 diagnosis becomes less certain and consequences for future offspring become more complex. Is the parent a case of reduced penetrance, genetic mosaicism or actually unaffected? When linkage analysis is performed in families diagnosed in this manner, results may be uninterpretable and cause anxiety in families. Therefore, we believe that an affected first degree relative should not be considered equally relevant for diagnosis. Particularly not in suspected adults, as the disease has a reported penetrance of virtually 100% at 6 years of age (10).

For that matter, genetic counseling of parents of an apparent de novo NF1 case may be even more difficult. The risk of germline mosaicism in one of the parents is difficult to determine. Once mutation analysis has reached full diagnostic power, it may resolve diagnostic problems in family members with insufficient (clinical) diagnostic criteria. However, establishing germline mosaicism will remain difficult, especially in mothers.

Our and other studies showed that more than one third of NF1-patients have one or more complications of the disease (5, 11). This group of patients as well as the group in which diagnosis is difficult, underline the functionality of multidisciplinary NF clinics as diagnostic and therapeutic centers, centers for referral and information, including facilities for genetic counseling and DNA-analysis. Centralization of care leads to extended experience which improves the quality of care in this complex disorder.

9.3 Therapeutic possibilities

In NF1, great psychological distress may be caused by excessive dermal neurofibromas and morbidity and mortality may be caused by plexiform neurofibromas and malignancies. Therefore, therapeutic developments focus on these anomalies. Until now, the only alternative therapy to surgery was laser therapy. However, no prospective studies have been reported on treament with laser beams. Experiments performed *in vitro* are focussing on restoration of the aberrant microtubule-mediated intracellular signal transduction pathway. In time, *in vivo* experiments in NF1 animal models may also be initiated. Although the mouse models heterozygous for an NF1 mutation do not show the major disease features of the disease, tumorigenesis is increased and learning disabilities are similar compared to those in humans (12, 13). Therefore, they may form suitable models for the effects of therapies on the tumor growth and intellectual functions.

As loss of both NF1 alleles activates *Ras* and causes unregulated cell growth, recovery of the signal transduction pathway may be established by drugs which reverse the oncogenic effects of activated *Ras*. In, for example, neurofibromin deficient Schwann cells, the main cell type in neurofibromas, this could lead to reversal of the phenotype. Normally, *Ras* proteins are farnesylated posttranslationally for membrane targeting and activation by farnesyltransferase (figure 9.1). Farnesyl protein transferase inhibitors (FPTI) have been shown to reverse *Ras*-transformed phenotypes in cell cultures and growth of malignant tumors in vivo (14). Recently, FPTI inhibited the growth of a neurofibrosarcoma cell line derived from an NF1-patient (15). In addition, FPTI was shown to reverse hyperplasia of NF1 deficient Schwann cells (16).
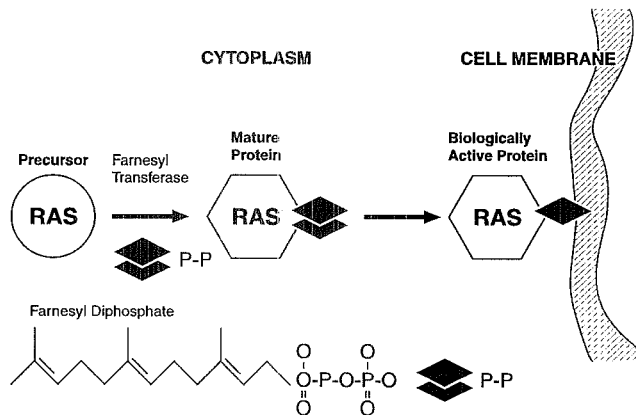

Figure 9.1
Posttranslational modification of RAS (courtesy Dr E. Legius)

Cell culture studies showed triggering of hyperplasia of NF1 deficient Schwann cells by addition of forskolin to the medium combined with a decrease in serum. Forskolin is an agent which increases cAMP in cells (16). This suggests that up-regulation of cAMP may also be involved in the growth of neurofibromas and downregulation may restrain growth.

In summary, recent experiments show that it may be possible to intervene in or bypass the signal transduction pathways mediated by neurofibromin. Although initial results are promising, therapeutic trials in humans are not expected in the near future.

9.4   The possibilities and limitations of linkage- and mutation analysis

As discussed, the low mutation detection rate in the NF1 gene is presumably due to the large size of the gene (the NF1 gene is one of the largest known human disease genes), in combination with the high frequency of small mutations and the paucity of known recurrent mutations. Therefore, it is impossible to screen the entire gene in every NF1-patient. In time, most of these obstacles will be overcome by advances in molecular genetic techniques. These may uncover mutational "hot spots" or an as yet unknown mutational mechanism, responsible for NF1 in the majority of patients. In addition, techniques that will enable automated analysis of large stretches of DNA, such as the DNA-chip technology, are rapidly developing (17).

Hypothetically, genetic heterogeneity may also play a role in the low detection of mutations. However, no evidence has been presented supporting this.

The benefits of linkage- and mutation analysis are the following. First, establishment of linkage or mutation detection enables (presymptomatic) diagnosis in familial cases and may lead to exclusion of the diagnosis in suspected individuals. Second, recurrent diagnostic problems, caused by the extreme clinical variability of NF1 may become solved. Examples are segmental Neurofibromatosis patients, or patients with insufficient diagnostic criteria such as the child with *café-au-lait* spots and JCML in chapter 8. NF1 diagnosis in the sibling and father formed a contraindication for a bone marrow transplant derived from these relatives.

In addition, prenatal diagnosis may be offered to NF1 families in which the intragenic polymorphic markers prove informative or a mutation has been found. In theory, preimplantation diagnosis is also possible in these families. The high probability of genetic mosaics within the NF1 population suggests that prenatal diagnosis may also be offered to parents of an apparent de novo NF1-patient with an identified mutation.

At this moment however, the low detection rate limits the possibilities of DNA-analysis in the NF1 gene. Once this has been overcome, general rules for careful application may be followed. Testing children under 16 years of age using mutation- or linkage analysis seems indicated only when there is a medical indication. In its absence, identification of a future disease of unknown prognosis is seen as inappropriate, also because a medical cure or prevention is not possible (18, 19).

At our department, since 1990, prenatal diagnosis was requested in 5 pregnancies from 4 families. An NF1-affected foetus was detected by linkage analysis in 3 cases; only one couple opted for termination (Dr. F.J. Los, personal communication). As prenatal diagnosis by linkage analysis may be offered to familial cases which form approximately one fifth of our population, it may be concluded that parents at risk for NF1 in their offspring, rarely requested prenatal testing. Also, in the case of an affected foetus, the pregnancy is not always terminated (20-22).

The extreme clinical variability and the low mortality of the disease presumably leads to the low uptake of both prenatal diagnosis (and preimplantation diagnosis) (23-25). It may be difficult to terminate the pregnancy of a child with a disease that a parent has learned to live with. In some cases, couples prefer to know the disease status of the foetus, so they can come to terms with an affected child and evade anxiety during the pregnancy.

Although most couples would not consider a prenatal test unless it could predict disease severity, our and other studies also show that the availability of prenatal diagnosis is greatly appreciated by NF1-patients and their families (chapter 6) (4, 26). This again demonstrates that the availability of prenatal diagnosis does not automatically lead to application by parents at risk for these diseases in their offspring.

9.5   Genotype-phenotype correlations

Now that a number of recurrent mutations or similar mutations (entire gene deletion, mutation in same codon) have been identified in familial and unrelated individuals, conclusions may cautiously be drawn on the viability of genotype-phenotype relationships.
As is illustrated in chapter 8, no correlations have been found between mutation type and phenotype. An exception are the entire gene deletions which show a tendency towards a severe phenotype of intellectual impairment, dysmorphic features with or without excessive dermal neurofibromas at a young age (chapter 7).

But even within this category, clinical variability is present (chapter 8). This may partly be explained by the extent of the deletions in these patients, which have not been specified in most cases. However, it is probable that when the boundaries of the deletions are established and neighboring genes have been identified, the phenotypic expression will remain variable, as is clearly expressed within NF1 families. Moreover, patients with mutations in specific regions of the NF1 gene have not shown similarities in severity of the phenotype. Deletion of- or mutations within the NF1-GRD of the NF1 gene (the only identified functional domain of the gene) has not resulted in a specific phenotype with excessive benign or malignant tumors as might have been suspected (27)(chapter 8). One NF1 missense mutation within the NF1-GRD, which was also detected in our study (chapter 8), was associated with a dramatic reduction in GAP activity (200 to 300-fold) (28). The patient in our study however, showed only dermatological symptoms of NF1. Patients with missense mutations in the same codon in exon 24, the most conserved exon, also showed no phenotypical similarities whatsoever (chapter 8).

In addition, it has been hypothesized that NF1-patients with JCML may have mutations affecting the intragenic genes EVI2A and EVI2B, the human homologues of murine genes involved in leukemia. However, such deletions were not found in NF1-JCML patients' lymphocytes or bone marrow (29). Also patients with both NF1 and multiple sclerosis (MS) were expected to have mutations disrupting *OMGP*, as *OMGP* encodes a glycoprotein involved in myelinization. Again, in the few NF1-MS cases studied these were not found (30).

However, this absence of straightforward genotype-phenotype relationships illustrates that the phenotype has a more complex molecular background. The well known phenotypic variability in many autosomal dominant diseases will become a source for studying complex and variable pathways within a regulatory system.

9.6   Conclusion

Since the cloning of the NF1 gene in 1990, progress has been made in both clinical and molecular genetic aspects of the disease. However, most clinical studies are still observational and little is known of the pathogenesis of NF1-related symptoms. In addition, mutation detection rates have remained low. Apparently, the full diagnostic potential of mutation analysis awaits the development of more advanced molecular genetic techniques. Therefore, it may be concluded that NF1 is still a clinical diagnosis in the majority of cases. Results of genotype-phenotype studies illustrate the complexity of this "monogenic" disease. Until we have elucidated the intricate interaction of the NF1 gene mutation, modifying genes and possible environmental

influences, mutation analysis and presymptomatic and prenatal diagnosis will only tell us the disease status of the individual but neither severity nor prognosis of the disorder.

Further analysis of the role of neurofibromin as a mediator in microtubule-mediated intracellular signal transduction pathways may, in time, explain and predict the clinical expression of the disease. In the meantime, clinical- and molecular genetic studies are indispensable for optimalization of patient management, establishment of the full extent of phenotypical variation, detection of possible risk factors and modifiers affecting prognosis and resolution of pre- and postnatal diagnostic problems in NF1.

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Chapter 10

Summary/ Samenvatting

Summary

This thesis presents clinical and molecular genetic studies in Neurofibromatosis type 1 (NF1) patients, including a prospective 10 year multidisciplinary follow-up study in 150 children with the disease and extensive genotype-phenotype studies in patients with entire NF1 gene deletions and identical mutations. This is the largest single centre study of NF1 at pediatric age until now.

Neurofibromatosis type 1 has an incidence of 1:2500 newborns. It is an autosomal dominant disease defined by the presence of multiple *café-au-lait* spots, skin fold freckling, neurofibromas, iris hamartomas (Lisch nodules), optic pathway glioma, distinctive osseous lesions and - in approximately half of the cases - a positive family history. The penetrance is virtually 100% at 6 years of age (1). Complications include disfigurement due to plexiform neurofibromas, optic pathway glioma, skeletal abnormalities, severe scoliosis and hemihypertrophy. Moderate to severe mental retardation is rare but various learning-, speech-, motor- and behavioral disabilities are more frequent. Other complications are endocrinological abnormalities, epilepsy, hypertension, and an increased risk of malignancies. The intra- and interfamilial clinical variability is extreme. In general, one third of NF1-patients have one or more severe complications of the disease (2-4).

Clinical aims of this study (chapter 1) included the analysis of the prevalence and incidence of symptoms and specific complications in 150 children with NF1 and evaluation of possible risk factors for the development of complications. This allowed further delineation of the clinical phenotype and its evolution. The functionality of a multidisciplinary NF1 clinic is demonstrated in the early diagnosis of NF1 and information of parents, relatives, general practitioners and medical specialists.

Molecular genetic aims (chapter 1) included the performance of linkage-and mutation analysis for diagnosis, including presymptomatic and prenatal diagnosis and genotype-phenotype studies. The NF1 gene was identified in 1990 on chromosome 17q11.2 and spans a region of about 350 kb of genomic DNA (5-7), containing approximately 60 exons (8).

In **chapter 2**, the results of a **10 year prospective multidisciplinary follow-up study** in 150 children with NF1 are presented (mean duration of follow-up: 4.9 years; SD = 3.8). Follow-up consisted of clinical examinations by NF1 specialists including a pediatrician, dermatologist, pediatric neurologist, ophthalmologist and clinical geneticist. Additional studies were performed as required. At first visit, clinical evaluations, X-rays of the cranium and the entire spine and

a visual evoked potential (VEP) were performed. Ninety five of the 150 children (63%) presented without complications (follow-up: 340.8 person years). Both prevalences and incidences of symptoms and complications were reported. In addition, possible risk factors for the development of complications were analyzed.

Most complications were already present at first presentation. During the observation period the percentage of children with complications rose from 37% to 41%. In children with NF1 presenting without complications, 2.4 complications developed per 100 person years. Complications presenting during follow-up were optic pathway gliomas, endocrinological abnormalities, malignancies, scoliosis and atlantoaxial dislocation. Interestingly, plexiform neurofibromas, usually assumed to be congenital, were also seen as newly occurring complications. An unexplained association was found between behavioral problems and the presence of complications.

Most important conclusions on patient management were that at first clinic visit, additional examinations of blood, urine or by X-ray, ultrasound, CT-scan or MRI may safely be performed not routinely but only on medical indication. Visual evoked potentials were considered a valuable addition to an ophthalmological examination at first presentation. Multidisciplinary follow-up examinations were recommended every 1 to 2 years in children without complications, unless problems arise earlier. Children with complications at presentation must be examined more frequently.

In **chapter 3**, the frequencies of the **minor disease features** are reported. Their value in children ≤ 6 years with a suspected diagnosis of NF1 was evaluated, considering that the disease is 100% penetrant at 6 years of age.

Macrocephaly (52.9%), **short stature** (24.7%), **hypertelorism** (63.5%) and **thorax abnormalities** (37.6%) were highly prevalent in NF1-affected children and significantly associated with a diagnosis of NF1 at 6 years of age. The mean number of minor disease features was significantly higher in children with NF1 at 6 years of age compared to those without a diagnosis at 6 years of age. Moreover, children with three or more minor disease features were all diagnosed with NF1 under the age of 6 years. Multivariate analysis using a logistic regression model showed that all minor disease features were independently associated with the presence of NF1 at 6 years of age. Therefore, in children with insufficient diagnostic criteria and ≤ 6 years of age, documentation of minor disease features may be a helpful aid in predicting the diagnosis of NF1 in years to come.

In 122 NF1-affected children, the prevalence of **endocrinologic disorders** and the relationship between these disorders and **cerebral abnormalities** on magnetic resonance imaging were analyzed (**chapter 4**). Central precocious puberty (CPP) was diagnosed in 3 children (2.5%) and growth hormone deficiency (GHD) in 3 children (2.5%). Optic pathway gliomas were observed in 15 children; in 9 of those, the optic chiasm was involved. Of the 3 children with CPP, only 1 had a concomitant chiasma glioma. In 1 case with GHD, an optic chiasm glioma was detected. Two of the 9 (22.2%) children with an optic chiasm glioma presented with CPP or GHD. In contrast to reported data (9), we found that CPP may develop in children in the absence of a chiasma glioma. This study also reports the first prevalence of 2.5% for GHD.

In **chapter 5**, a case of **atlantoaxial dislocation** in a child with Neurofibromatosis type 1 and cervical dysplasias is described manifesting after surgery for a plexiform neurofibroma in the head/neck region. The number of cervical vertebral complications associated with Neurofibromatosis type 1 (11.8%) (4) and the perioperative risks of abnormal C1-C2 mobility makes this complication an important point in the clinical and perioperative management of NF1-cases by the neurologist, neurosurgeon, radiologist and anesthesiologist. Complete bidirectional cervical vertebral column X-rays are recommended in all NF1-patients undergoing anesthesia and/ or surgery. If abnormalities are observed and intubation is required, a supplementary flexion-extension test can be obtained to assess the perioperative risk.

Intubation may be performed under fixation of the cervical column or with fibrescopic guidance. The presence of cervical dysplasias or spinal neurofibromas may also be a reason to opt for regional anesthesia if feasible.

Chapter 6, evaluates the **diagnostic delay** observed in NF1-patients and their affected family members before analysis by the multidisciplinary NF clinic. Furthermore, the information on the disease at diagnosis, preference for an early diagnosis, and the general attitude towards prenatal diagnosis were evaluated. Half of the affected children and one third of the affected adults were treated for symptoms related to NF1 before a specific diagnosis was made. Parents preferred an early diagnosis of NF1 in their children. One third of the parents of NF1-affected children and 58% of the affected adults were not satisfied with the information provided at the time of the diagnosis. The pediatrician, clinical geneticist and the patient support group were considered the most important sources of information on the disease. The majority of parents with a risk for NF1 in their future offspring did not refrain from having children. The general attitude towards prenatal diagnosis was positive; however few parents actually request termination of an affected pregnancy, as our and international experience shows.

In **chapters 7** and **8**, the results of **mutation analysis in 200 unrelated NF1-patients** are presented. Patient DNA was screened for large deletions by microsatellite analysis and for small mutations by SSCP analysis. Subsequently, large deletions were delineated by Southern analysis and smaller mutations were identified by sequence analysis and confirmed by ASO hybridization. Despite the development of new techniques mutation detection rates remain low: between 10-20% of clinical series (10). Most important reasons for this seemingly low detection rate are the large size of the gene, the high frequency of small mutations and the paucity of recurrent mutations. The mutations found in our series **contribute almost 10%** to those reported to the NF1 Genetic Analysis Consortium (11).

Eight **large deletions** were found, 6 of which were shown to comprise the entire NF1 gene. All but one showed a severe phenotype of mental retardation, dysmorphic features and early onset of multiple dermal neurofibromas. The deletion was of maternal origin in 5 cases and of paternal origin in 1 case, in 2 cases it was undetermined. The total of entire gene/ large deletions reported is 27, and a mild phenotype without mental retardation or severe learning disabilities is observed in 3 cases.

Small mutations were detected in 11 and possibly 12/192 (6%) of NF1-patients after screening of approximately 12% of the NF1 coding region (exons 2, 24, 26, 28 and part of 31). Besides 6 and possibly 7 novel mutations, the recurrent mutations R1947X and K1423E were detected in 4 families (11 affected individuals) and in 1 case. The phenotypes observed in these patients illustrate the large intrafamilial and interfamilial variability in phenotypic expression between NF1-patients with identical or similar mutations.

Chapter 9 presents **conclusions** from these studies for diagnosis, understanding and management of NF1. Observational studies in specialized NF centers remain important as NF1 is still a clinical diagnosis in the majority of cases, and molecular or biochemical indicators for progression of the phenotype are lacking. The extreme clinical variability in patients with identical or similar mutations demonstrates the complex molecular nature of the phenotype, involving modifying genes and possibly environmental factors. Future studies focusing on modifiers are of critical importance for further understanding of the pathogenesis and attempts at therapeutic interventions.

Samenvatting

Dit proefschrift beschrijft zowel klinisch als moleculair genetisch onderzoek van Neurofibroma-tosis type 1 (NF1) patiënten, waaronder een 10-jarig vervolgonderzoek van 150 kinderen die regelmatig onderzocht werden door een multidisciplinaire werkgroep (kinderarts-coördinator, kinderneuroloog, dermatoloog, en oogarts). De erfelijke eigenschap betrokken bij NF1 werd op afwijkingen (mutaties) onderzocht en deze veranderingen in het erfelijk materiaal (genotype) werden gerelateerd aan de klinische verschijnselen van een individu (phenotype). Het onderzoek naar symptomen en complicaties bij kinderen met NF1, is het grootste onderzoek tot nu toe gepubliceerd.

Neurofibromatosis type 1 komt voor bij 1:2500 mensen. Het is een autosomaal dominant ziekte: een aangedane ouder heeft 50% kans om de ziekte aan een kind door te geven. In de helft van de gevallen is de patiënt de eerste in de familie met de afwijkende eigenschap. De ziekte wordt gekenmerkt door koffie met melk-kleurige vlekken (*café-au-lait* vlekken), sproetjes in de lichaamsplooien, goedaardige gezwellen uitgaande van de cellen die zenuwen omhullen (neuro-fibromen) en gepigmenteerde bolletjes op het regenboogvlies (Lisch noduli). Hoewel de *café-au-lait* vlekken veelal bij de geboorte aanwezig zijn, verschijnen de overige karakteristieke symptomen met toenemende leeftijd. Echter, de meerderheid van de patiënten kan vóór het zesde levensjaar gediagnostiseerd worden (1). Hoe de ziekte zich zal ontwikkelen is niet te voorspellen, ook niet binnen één familie.

Ernstige complicaties worden in ongeveer een derde van de patiënten met NF1 gezien (2-4). Deze zijn: misvormingen ten gevolge van plexiforme neurofibromen, goedaardige gezwellen van de oogzenuw (opticus glioom) die soms leiden tot verlies van het gezichtsvermogen, skeletafwijkingen, ernstige rug verkrommingen en een toename in omvang van een ledemaat of lichaamshelft (hemihypertrofie). Maar ook, afwijkingen in hormoonhuishouding, epilepsie, hoge bloeddruk en kwaadaardige gezwellen worden vaker gezien dan in de algemene bevolking. Milde tot ernstige mentale retardatie is zeldzaam. Daarentegen komen leerproblemen in 30-45% van NF1-patiënten voor. Ook vertonen zij vaker spraak-, motorische- en gedragsproblemen.

De klinische doelstellingen van dit onderzoek (hoofdstuk 1) waren: een analyse van de **prevalentie en incidentie** van **symptomen en complicaties** bij 150 kinderen met NF1 tijdens een 10-jarige periode, en een evaluatie van mogelijke risico factoren voor het ontwikkelen van complicaties. Het doel was een duidelijker beeld te krijgen van het verloop van de klinische verschijnselen van de ziekte. Het praktische **nut van een multidisciplinaire werkgroep NF** werd

aangetoond door een vroegere diagnose van NF1 en betere informatie van ouders, familieleden, huisartsen en medische specialisten.

De moleculair genetische doelstellingen (hoofdstuk 1) waren: het verrichten van linkage- en mutatie onderzoek voor presymptomatische en prenatale diagnostiek en het nagaan van verbanden tussen genotype en phenotype. Het NF1 gen werd geïdentificeerd in 1990 en bevindt zich op chromosoom 17q11.2. Het is een zeer groot gen (350 kb) (5-7) dat bestaat uit ongeveer 60 coderende gedeelten (exonen) (8).

Hoofdstuk 2 beschrijft de resultaten van een 10-jarig vervolgonderzoek van 150 kinderen met NF1 (gemiddelde follow-up duur: 4.9 jaar; SD = 3.8). Bij de regelmatige poliklinische bezoeken werden de kinderen gezien door een kinderarts, kinderneuroloog, dermatoloog, en oogarts, allen met ervaring op het gebied van NF1. Aanvullend onderzoek werd verricht op medische indicatie. Bij het eerste polikliniek bezoek werden röntgenfoto's gemaakt van de schedel en de gehele wervelkolom om botafwijkingen behorend bij NF1 op te sporen. Onderzoek naar reacties van het oog, afgeleid van de hersenen (visual evoked potential, VEP) werd verricht om opticus gliomen (gezwellen van de oogzenuw) vroeg te kunnen opsporen.

Vijfennegentig van de 150 kinderen (63%) hadden geen complicaties bij hun eerste bezoek (follow-up duur: 340.8 persoonsjaren). Zowel prevalenties als incidenties van symptomen en complicaties werden in deze studie berekend. Ook werden mogelijke risico factoren voor het ontwikkelen van complicaties geëvalueerd.

Het merendeel van de complicaties waren aanwezig bij het eerste bezoek. Tijdens de follow-up periode steeg het percentage kinderen met complicaties van 37% naar 41%. Bij kinderen zonder complicaties bij aanmelding ontwikkelden zich 2.4 complicaties per 100 persoonsjaren. Het betrof hierbij: opticus gliomen, endocriene afwijkingen, kwaadaardige gezwellen, een ernstige rugverkromming en een verschuiving van de eerste ten opzichte van de tweede halswervel (atlantoaxiale dislocatie). Plexiforme neurofibromen, worden over het algemeen als aangeboren beschouwd, maar werden in dit onderzoek bij een aantal kinderen pas tijdens follow-up opgemerkt. Er werd een onverklaarbare associatie gevonden tussen gedragsproblemen en de aanwezigheid van een of meer complicaties.

De resultaten van het onderzoek hadden gevolgen voor het beleid ten aanzien van jonge NF1-patiënten. Aanvullend onderzoek is alleen geïndiceerd op medische indicatie. VEP onderzoek is echter een goede aanvulling op het uitgebreid oogheelkundig onderzoek bij het eerste bezoek.

Controles door een multidisciplinaire NF-team worden aanbevolen elke 1 à 2 jaar bij kinderen met NF1 zonder complicaties. Kinderen met complicaties dienen frequenter te worden gezien.

In **hoofdstuk 3**, worden de **prevalenties van typische**, maar niet specifieke **kenmerken bij NF1-patiënten** besproken zoals een grotere schedelomtrek (macrocephalie), een kortere gestalte, wijder uit elkaar staande ogen (hypertelorisme) en een afwijkende vorm van de borstkas. Deze kenmerken blijken van waarde te zijn bij de diagnose van kinderen $\leq$ 6 jaar die onvoldoende diagnostische hoofdkenmerken van NF1 hebben.

Macrocephalie (52.9%), **een kortere gestalte** (24.7%), **hypertelorisme** (63.5%) **en borstkas afwijkingen** (37.6%) waren frequenter bij kinderen die gediagnostiseerd waren op 6-jarige leeftijd dan bij ongediagnostiseerde kinderen van die leeftijd. Het gemiddeld aantal typische doch niet specifieke kenmerken was ook significant hoger in kinderen gediagnostiseerd op 6-jarige leeftijd vergeleken met ongediagnostiseerde kinderen van 6 jaar. Kinderen met 3 of meer van deze kenmerken, waren allen gediagnostiseerd op 6-jarige leeftijd. Multivariate analyse met behulp van een logistische regressie model liet zien dat deze eigenschappen allen onafhankelijk geassocieerd waren met NF1 diagnose op 6-jarige leeftijd. Geconcludeerd werd dat deze kenmerken van waarde kunnen zijn bij de diagnostiek bij kinderen $\leq$ 6 jaar.

Bij 122 kinderen met NF1 werd de prevalentie van **endocriene afwijkingen** en hun verband met afwijkingen op MRI-beeldvorming van de hersenen bepaald (**hoofdstuk 4**). Te vroeg beginnen van de puberteit (centrale pubertas praecox, CPP) werd gezien bij 3 kinderen (2.5%) en een groeihormoon tekort (deficientie, GHD) bij 3 kinderen (2.5%). Opticus gliomen werden gevonden bij 15 kinderen; bij 9 van hen was er een glioom in het chiasma opticum (de overkruisingsplaats van de oogzenuw). Van de 3 kinderen met CPP, had er één tevens een chiasma glioom. Bij één kind met GHD, werd een chiasma glioom gedetecteerd. In totaal, ontwikkelden 2 van de 9 kinderen met een chiasma glioom (22.2%) CPP of GHD. In tegenstelling tot eerdere veronderstellingen (9) blijkt CPP zich te kunnen ontwikkelen in afwezigheid van een chiasma glioom. Tevens werd voor het eerst een prevalentie van GHD bij kinderen met NF1 gerapporteerd (2.5%).

In **hoofdstuk 5**, wordt een casus van een kind met NF1 beschreven met afwijkingen aan de halswervels, die een atlantoaxiale dislocatie (beklemming van het ruggemerg door verplaatsing van de atlas en de draaier in de nek) ontwikkelde tijdens een operatie van een plexiform neurofibroom in de hals. Het hoge percentage afwijkingen aan de hals wervels bij NF1 (11.8%) (4) en de risico's van een atlantoaxiale dislocatie (mogelijke dwarslaesie) rechtvaardigen een preventief beleid bij NF1-patiënten die een operatie moeten ondergaan. Het wordt aanbevolen

vóór de operatie röntgenfoto's van de halswervels in twee richtingen te maken. Wanneer er afwijkingen worden gezien en intubatie (inbrengen van beademingsbuis) voor de operatie nodig is, zal aanvullend een flexie-extensie test (heen en weer buigen van het hoofd onder röntgendoorlichting en onder controle van de reacties van het zenuwstelsel) worden verricht om het risico van de operatie in te schatten. Vaak zal het mogelijk zijn te intuberen opgeleide van een buigzame fibrescoop. Afwijkingen aan de halswervels als mede plexiforme neurofibromen in de wervelkolom kunnen een reden zijn om regionale anesthesie toe te passen.

Vertraging in het stellen van de diagnose bij NF1-patiënten en hun familieleden, voorafgaand aan het bezoek aan een multidisciplinaire NF team is een veel voorkomend probleem (**hoofdstuk 6**). De helft van de kinderen en een derde van de volwassenen werden reeds behandeld voor klachten veroorzaakt door NF1 vóórdat die diagnose gesteld werd. Een derde van de ouders van kinderen met NF1 en 58% van de aangedane ouders waren ontevreden over de verstrekte informatie bij diagnose. De kinderarts, de klinisch geneticus en de patiëntenvereniging werden gezien als de belangrijkste bronnen van informatie. Ouders gaven de voorkeur aan een vroege diagnose van NF1, ook wanneer er geen complicaties waren. De meerderheid van de aangedane ouders vond NF1 geen reden om van kinderen af te zien. De algemene houding ten opzichte van prenatale diagnostiek was positief. Echter, weinigen zouden een zwangerschap beëindigen van een aangedane vrucht, zoals ook in andere studies is vastgesteld.

In **hoofdstukken 7 en 8**, worden de resultaten gepresenteerd van **mutatieanalyse in 200 indexpatiënten met NF1**. Het erfelijk materiaal (DNA) werd onderzocht op het ontbreken van grote stukken gen met behulp van polymorfe markers (stukjes DNA die binnen de bevolking zeer variabel zijn). Kleinere veranderingen werden onderzocht met SSCP analyse (techniek gebaseerd op veranderde mobiliteit van stukjes DNA met een verandering). De grotere deleties werden nader gespecificeerd door middel van Southern blotting (techniek waarbij intensiteitsveranderingen worden gemeten van aan het DNA toegevoegde probes). Kleinere mutaties werden geïdentificeerd door volgorde bepaling van het betrokken deel en bevestigd door ASO hybridisatie (controleren van verandering d.m.v het toevoegen van een stukje sequentie met de gevonden verandering en een stukje met de normale volgorde van het DNA ter plekke). Ondanks nieuwe technieken is vaststellen van een kleinere of grotere DNA verandering mogelijk bij 10-20% van NF1-patiënten (10). Belangrijkste oorzaken daarvan zijn de grootte van het gen, het weinig voorkomen van eenzelfde mutatie en de hoge frequenties kleine mutaties. De mutaties in deze studie vormen **bijna een tiende deel** van de bij het "NF1 Genetic Analysis Consortium" aangemelde mutaties (11).

Acht grote deleties, werden gevonden; in 6 was minimaal het gehele gen afwezig. Bij 7 van de 8 patiënten werd een typisch uiterlijk gezien van mentale retardatie of ernstige leerstoornissen en dysmorfe kenmerken. De deletie was afkomstig van het moederlijk erfelijk materiaal bij 5 individuen en van de vader in een patiënt, in 2 gevallen kon de herkomst niet worden geëvalueerd. Totaal zijn in de literatuur 27 grote deleties bekend. Een mild phenotype zonder mentale retardatie of ernstige leerproblemen en dysmorfe kenmerken werd bij slechts 3 patiënten waargenomen.

Kleinere mutaties werden in 11 patiënten en mogelijk 12/192 (6%) NF1-patiënten gedetecteerd na screening van ongeveer 12% van het coderende gedeelte van het NF1 gen (exonen 2, 24, 26, 28 en deel van 31). Naast 6 en mogelijk 7 nieuwe mutaties, werden de bekende mutaties R1947X en K1423E gedetecteerd in 4 families (11 aangedane individuen) en in een patiënt met een nieuwe mutatie. De klinische verschijnselen bij deze patiënten onderstrepen de grote klinische variabiliteit van het ziektebeeld, zowel binnen families, als tussen patiënten met eenzelfde mutatie.

Hoofdstuk 9 presenteert de **conclusies** van dit onderzoek met betrekking tot de **diagnostiek, therapie en het medisch beleid** bij de NF1-patiënt. Beschrijvende studies in gespecialiseerde NF-klinieken blijven belangrijk aangezien NF1 in de meeste gevallen nog steeds een klinische diagnose is en moleculair genetische of biochemische parameters voor progressie nog ontbreken. De extreme variabiliteit in patiënten met identieke dan wel gelijksoortige mutaties illustreert de complexe moleculaire achtergrond van de ziekte, waarbij mogelijk andere genen en omgevingsfactoren een rol spelen. Toekomstige studies die zich richten op de modificerende genen zijn belangrijk voor een verder begrip van het ziektebeeld, het verloop en mogelijke therapeutische interventies.

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Curriculum Vitae

Marjon Hester Cnossen born in Washington D.C., U.S.A. on May 23, 1966.

1978 - 1984   Grammar School: Christelijk Gymnasium "Sorghvliet" in the Hague, the Netherlands

1985 - 1990   Doctoral Exam in Medicine (Medical Faculty of Leiden University)

Electives:
Cell Biology and Histology

Research subjects:
Bronchitis and asthma in children in the region of El Kef, Tunesia.
(Prof. Dr K.F. Kerrebijn, Prof. Dr J.P. Vandenbroucke, Prof. Dr Ph. Quanjer, Dr P.J. van der Straaten).

Follow-up study on results of the Belsey Mark IV operation.
(Prof. Dr H.G. Gooszen, Dr J.M.L.M. Horbach).

Diffuse Intravasal Coagulation (DIC) in abortus provocatus after 16 weeks.
(Prof. Dr A. Peters, Dr F.W. Jansen).

Prognostic value of neonatal patterns of movement.
(Dr L. den Ouden).

Foreign Internships:
Pediatrics and Neonatology
(Doküz Eylul Hospital and Egé University Hospital in Izmir, Turkey)

1990 - 1991   First-year Exam Art History (Art Faculty of Leiden University)

1991 - 1993   Medical Degree, cum laude (Medical Faculty of Leiden University)

1993 - 1997   Clinical and Molecular Genetic studies on Neurofibromatosis type 1 (NF1) (PhD thesis)

Courses:
Principles of Research in Medicine and Epidemiology, Clinical and Population Genetics, Genetic Epidemiology, Clinical Epidemiology on Schiermonnikoog, Introduction to Biostatistics, Classical Methods for Data Analysis, Regression Analysis, Congenital Malformations, Risk Analysis in Laboratories, Oxford examination in English as a foreign language, highest level

1996   Counselor at the Internationaal Summer Camp for children with Neurofibromatosis

1998 -   Residency in Pediatrics, Sophia Children's Hospital, Rotterdam
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Marjon is married to Tadek Hendriksz and they have a daughter Marijn Stasia.

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submitted for publication.

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Stellingen

1. Ook kinderen met Neurofibromatosis type 1 (NF1) zonder complicaties zijn gebaat met regelmatige controles door een gespecialiseerd medisch team.
   Dit proefschrift.

2. Bij kinderen met NF1 en een centrale pubertas praecox kan de endocrinologische stoornis niet altijd worden verklaard door de aanwezigheid van een glioom van het chiasma opticum.
   Dit proefschrift.

3. Kinderen met NF1 hebben vaker groeihormoondeficiëntie dan gezonde kinderen, en wel met een vastgestelde prevalentie van 2.5%.
   Dit proefschrift.

4. Consultatiebureauartsen kunnen een belangrijke rol spelen in de vroege diagnosestelling van NF1-patiënten, indien er in hun (na)scholing voldoende aandacht is voor de symptomen van NF1.
   Dit proefschrift.

5. Het ontbreken van een directe genotype-phenotype relatie bij mutaties betrokken bij dominant erfelijke ziekten, duidt vaak op een complexe functie of regulatie van het normale genproduct.
   Dit proefschrift.

6. Nu door vaccinatie van kinderen meningitis door *H. Influenzae* type b is afgenomen, komt bacteriële meningitis vooral bij volwassenen voor.
   Schuchat et al. N Engl J Med 1997;337:970-6.

7. Patiënten met sepsis behandeld met ibuprofen hebben lagere prostacycline en thromboxane spiegels en minder koorts, tachycardie, zuurstof verbruik en lactaat acidose. Echter, het optreden van shock, "acute respiratory distress syndrome" en de overleving worden er niet door beïnvloed.
   Bernard et al. N Engl J Med 1997;336:912-8.

8. Diagnostiek van erfelijke ziekten bij kinderen dient allereerst in het gezondheidsbelang van het kind te zijn.
   Fanos. Am J Hum Genet 1995;57:1233-41.

9. Als vrouwelijke wetenschappers 2.5 maal zo goed moeten zijn als hun mannelijke collegas om gelijk beoordeeld te worden, dan is het een extra kapitaalvernietiging hun niet in staat te stellen langdurig part-time te kunnen werken.
Wenneras et al. Nature 1997;387:341-3.

10. De maatschappelijke onrust over het versterven wordt grotendeels veroorzaakt doordat de hiervoor (door de medische wereld) gekozen benaming de suggestie geeft van een handeling.

11. "Children are a country's greatest natural resource."
Danny Kaye

12. Als wordt aangetoond dat er een relatie bestaat tussen geweld op televisie en geweld op straat, moet de regering beleid ontwikkelen dat de jeugd aanmoedigt de straat op te gaan.

13. Hoewel het doen van promotieonderzoek soms voelt als het koesteren van een kind, voelt het opvoeden van een kind nooit als het uitvoeren van promotieonderzoek.

Stellingen behorende bij het proefschrift "Neurofibromatosis type 1: A Clinical and Molecular Genetic Study" van Marjon H. Cnossen. Rotterdam, 17 december 1997.