COGNITIVE DEFICITS IN CHILDREN WITH NEUROFIBROMATOSIS TYPE 1:

FROM RECOGNITION TO TREATMENT.

COGNITIEVE PROBLEMEN BIJ KINDEREN MET NEUROFIBROMATOSE TYPE 1:

VAN HERKENNING TOT BEHANDELING.

LIANNE CAROLINE KRAB

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CHAPTER 1

Introduction, Aims and Outline

Introduction

Over the past few years, mouse models have significantly contributed to our understanding of the molecular mechanisms underlying cognitive dysfunction in genetic disorders. Moreover, several preclinical studies in mouse models of for instance Neurofibromatosis type 1 (NF1), Tuberous Sclerosis Complex, Down syndrome, Rett syndrome, and Fragile X syndrome have provided evidence that some of these cognitive deficits may be reversible by targeting the underlying molecular disturbances.[1-5] These new findings have sparked a great interest in the search for drugs that may be used in patients to ameliorate their cognitive problems.[6] A recent study described the beneficial effects of a statin, one of the most widely prescribed classes of medications, on cognitive deficits of a mouse model for NF1.[7] This finding offered an exciting and unique opportunity to assess the effect of a drug that has been validated in preclinical studies and for which substantial clinical safety data is available, on cognitive problems in NF1 patients.

This thesis focuses on the recognition and treatment of cognitive problems in children with NF1. It aims to provide an overview of the specific aspects of cognitive performance that affect daily life functioning in NF1 children, and tries to identify possible outcome measures that can be used to assess potential therapeutic interventions. This knowledge was used to perform the first randomized, double blind, placebo-controlled trial to assess the effect of statins on cognitive problems in children with NF1.

Neurofibromatosis type 1

Neurofibromatosis type 1 is an autosomal dominant disease with a birth incidence of about 1 in 3000, half of which are sporadic cases.[8] It is caused by a heterozygous mutation in the gene encoding the neurofibromin protein on chromosome 17q11.2.[9, 10] NF1 patients display characteristic neurocutaneous abnormalities, such as café-au-lait macules and neurofibromas, and have an increased incidence of malignant tumor formation. NF1 can affect physical functioning and appearance as well as cognitive performance and behavior.

Clinical manifestations of NF1

Clinical features of NF1 arise predominantly from neural crest derived tissues. The NF1 diagnosis is a clinical diagnosis, based on the presence of two or more major disease features, such as *café-au-lait* macules, axillary or inguinal freckling, and neurofibromas (see table 1 and

figure 1).[11] Minor disease features of NF1 include macrocephaly, hypertelorism, thorax deformities and small stature.[12] The manifestations of NF1 develop with age, but usually the diagnosis can already be made before the age of six.[8]

Table 1: NIH-defined diagnostic criteria for NF1, with their frequency and typical age of onset

Criterion[11]	Frequency[13]	Typical age of onset
6 or more *café-au-lait* macules (>0.5 cm in children or >1.5 cm in adults)	>99%	Congenital
2 or more neurofibromas, *or*	>99	> 7 y
one plexiform neurofibroma	30-50%	Congenital
Freckling in the axillary or inguinal region	85%	> 3 y
Optic pathway glioma	15%	< 7 y
2 or more Lisch nodules (iris hamartomas)	90-95%	> 7 y
Bony dysplasia, with or without bowing or pseudoarthrosis	±3%	Congenital
First degree relative with NF1	50%	Not applicable

The phenotype of NF1 is very variable, even within families. Although some patients only have *café-au-lait* macules, Lisch nodules and a few neurofibromas, other patients can display serious complications. Frequent complications at pediatric age include disfigurement due to plexiform neurofibromas, orthopedic problems (pseudoarthrosis (2%), scoliosis (10%)), endocrinologic problems (5%; including precocious or delayed puberty and growth hormone deficiency), cardiovascular problems (including pulmonary stenosis, and renal artery stenosis associated with hypertension (2%)), and malignancy (including optic pathway gliomas (15%, see figure 1), Juvenile myelomonocytic leukemia, low-grade central nervous system astrocytomas (2-3%), malignant peripheral nerve sheet tumors (life-time risk 8-13%), and phaeochromocytoma (2%)).[13-19] The most common complication to affect quality of life in children with NF1, however, are cognitive impairments,[20] including mental retardation (4-8%), specific neuropsychological deficits, learning disabilities and behavioral problems.[21] The unpredictable and diverse phenotype of NF1 stresses the importance of age-specific monitoring by NF1 specialists.[13]

Genetic background

NF1 is caused by a heterozygous mutation in the gene encoding for the neurofibromin protein on chromosome 17q11.2.[9, 10] The NF1 gene spans a region of about 335 kb of genomic DNA and consists of over 60 exons. Several exons are alternatively spliced, including exon 9a and 23a.

The neurofibromin isoform with exon 9a is exclusively expressed in postmitotic forebrain neurons,[22] whereas the isoform containing exon 23a is expressed predominantly in glial cells.[23]

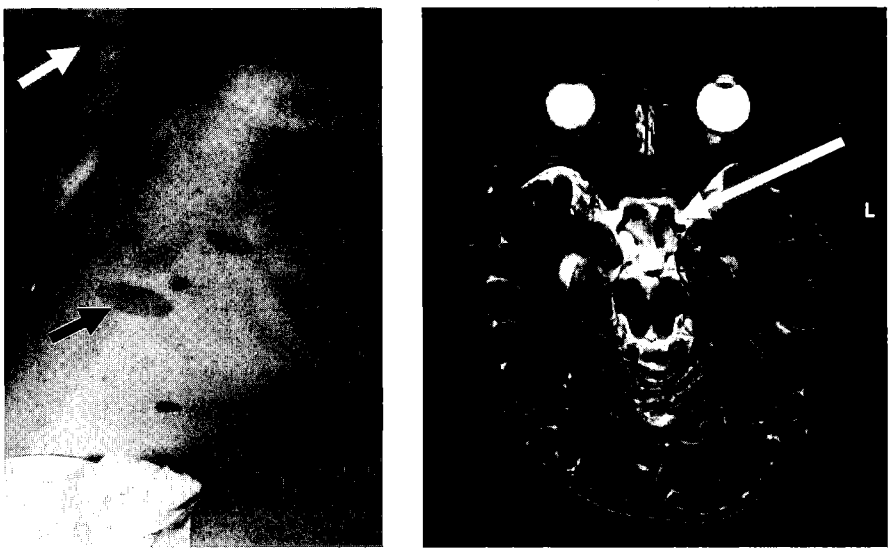

Figure 1. Clinical features of Neurofibromatosis type 1 (NF1).

Left: A toddler with familial NF1 with multiple *café-au-lait* maculae (dark arrow). Note the neurofibroma on the wrist of its mother (white arrow). *Right:* Transversal T2 weighed MR (Magnetic Resonance) image of a glioma of the optic chiasm (white arrow) in a 9-year old NF1 patient.

The mutation rate of the NF1 gene is about 10-fold higher than that of other disease genes, most probably because of its large size.[24] The spectrum of NF1 is very broad, with hundreds of individual mutations identified so far, distributed over the different exons of the NF1 gene. There are no clear mutational hotspots,[24, 25] although several recurrent mutations and mutation rich exons have been identified, together accounting for up to 30% of the mutations.[25] About half of all NF1 mutations result in premature termination codons,[24] 20-30% in splice defects, and approximately 10% of the mutations are missense or single amino acid deletions.[24, 25] About 5% of the NF1 patients have a microdeletion encompassing the entire NF1 gene and several flanking genes, which is associated with a more severe cognitive and physical phenotype.[26]

Neurofibromin

The NF1 gene encodes for neurofibromin, a 2,818 amino acid protein which is expressed in a wide array of cell types in the body, but is most abundant in neurons, Schwann cells and

oligodendrocytes.[27] Neurofibromin contains a GTP-ase activating protein (GAP) related domain, which spans about 10% of the protein sequence.[28] Through the GAP domain, neurofibromin acts as a negative regulator of the activity of the RAS (rat sarcoma viral oncogene homolog) proto-oncogenes. Thereby, neurofibromin functions as a tumor suppressor, which is illustrated by the finding that benign and malignant tumor cell lines of NF1 patients exhibit a decrease or loss of neurofibromin.[29-33]

By its action on RAS, neurofibromin downregulates the RAS/ERK (Extracellular signal regulated kinase) pathway[34] and the RAS-PI3K (Phosphoinositide 3-kinase) / MTOR (mammalian target of rapamycin) pathway.[35] In addition, neurofibromin modulates the cAMP (cyclic adenosine monophosphate) / PKA (cyclic AMP-dependent protein kinase A) pathway by regulating Adenylyl Cyclase function in both RAS dependent[36] and RAS independent ways.[37-41] A simplified overview of the actions of neurofibromin is provided in figure 2.

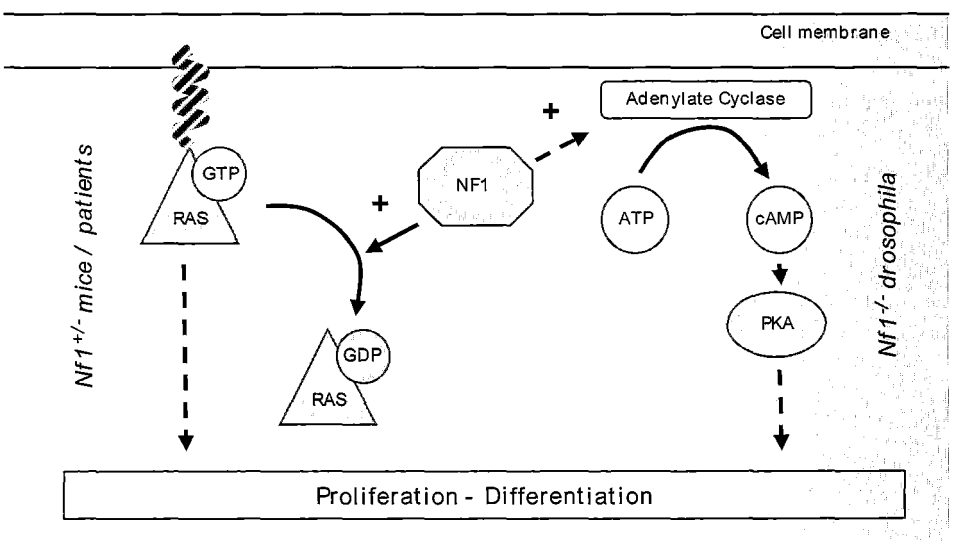

Figure 2: Simplified overview of the functions of neurofibromin. Evidence for action on the RAS pathway originates predominantly from research in heterozygous *Nf1* knockout mice and patient material, whereas evidence for the action on the cAMP/PKA pathway originates mostly from research in homozygous *Nf1* knockout drosophila.

A broad range of other properties and functions have been attributed to neurofibromin, including an association with microtubuli,[42] a possible involvement in vesicle transport via its interaction with Amyloid Precursor Protein,[43] and a role in actin filament reorganisation,[44] filopodia and dendritic spine formation[45], regulation of glial proliferation and neuronal differentiation,[38] and somatosensory cortex barrel formation.[46] Like many other tumor

suppressors, neurofibromin is targeted to the nucleus.[47] However, these functions are outside of the scope of this thesis.

Relationship to other disorders

NF1 shows remarkable phenotypical overlap with other diseases grouped under the Neuro-Cardio-Facial-Cutaneous (NCFC) syndromes and the Hamartoma syndromes, which include for instance Noonan Syndrome, Costello Syndrome, and Tuberous Sclerosis Complex. These diseases all involve some degree of cognitive impairment, cardiac defects, typical facial dysmorphisms, macrocephaly, and cutaneous abnormalities. Most of these disorders are associated with an increased risk of developing malignancies.[48, 49] The large overlap in clinical phenotypes and the frequent lack of definite diagnostic criteria can make it difficult to establish a diagnosis, especially in young children, when the disease phenotype is often not fully developed. Recent advances in clinical genetics have revealed the NCFC and Hamartoma syndromes are all associated with mutations in genes in the RAS/ERK and RAS/PI3K/MTOR pathways.[48, 49] Strikingly, despite the large overlap in genetic background between these diseases, even patients with identical mutations can display remarkably different phenotypes.[50] [51] Insights into how the mutations found in NCFC and Hamartoma syndrome patients affect neuronal signaling can facilitate the search for possible targeted treatments to alleviate the cognitive burden of these syndromes.

Cognitive problems in NF1

Neuropsychological profile

The mean IQ of NF1 patients is shifted to the left compared to the general population and sibling controls, and ranges from the high 80's to the low 90's.[20, 52-60] As a result, NF1 patients have a two-fold increased risk at mental retardation (IQ below 70; 4-8%) compared to the general population.[21]

In addition to a lower IQ, NF1 is characterized by impairments across multiple neuropsychological domains (thoroughly reviewed in [52, 61-63]). Deficits in visual spatial and visual constructive skills, especially on the Judgment of Line Orientation Test, have long been considered a hallmark of NF1.[20, 58, 64-66] Other affected domains include executive functions (such as planning and organization, and abstract concept formation), attention (divided, switching and sustained), language (expressive and receptive) and memory (verbal, nonverbal and tactile).[20, 54, 56, 57, 67-71] However, problems with nonverbal and tactile memory could be

secondary to poor visual spatial skills or tactile perception, and not all reports confirm the deficits in verbal memory.[63] Several studies indicate that the problems in attention, visual spatial skills and planning remain after correction for IQ.[20, 57]

Learning disabilities

Numerous studies have reported deficits in academic achievement tests administered in the neuropsychological test setting, compared to normative scores or controls.[54, 56-58, 65-68, 72-74] Learning disabilities are reported in all academic areas, including reading, spelling and mathematics (reviewed by Levine et al.[62]). Initial reports suggested the cognitive profile of NF1 resembles that of children with nonverbal learning disorder,[52] which is characterized by problems with motor behavior, social interaction, visual spatial skills and arithmetic, but not in language.[75] Although there are many parallels, this description does not quite fit. For instance, literacy based learning disabilities turn out to occur at least as frequently as mathematical problems in NF1.[54, 67, 73]

Estimates for the prevalence of learning disabilities vary considerably (between 35 and 70%),[21, 72] and suffer from small population sizes, selection bias, lack of control groups and differences in the definitions for learning disability.[52] According to the DSM-IV criteria, a Specific Learning Difficulty can be diagnosed only if academic achievement is more than two standart deviations below the individual's level of intelligence (IQ).[76] However, in NF1, low academic achievement is frequently seen in combination with a lower IQ.[20, 56, 72] Thus, by only acknowledging learning difficulties when patients show significant discrepancies between their academic achievement and IQ, we would seriously underestimate the actual problems in learning experienced by NF1 children.[77]

There is still little information about how NF1 children function at school, where their cognitive skills are put to the test in a setting that is in many ways different than the neuropsychological test setting. Receiving intensive remedial teaching or special education may seriously confound the interpretation of academic achievement test scores, and this may result in a significant underestimation of the learning disabilities and school problems associated with NF1. Therefore, to get a more realistic assessment of school performance, it is important to combine these different types of information on school functioning. However, quantitative studies on the level of special education, remedial teaching, or grade repetition in these children are largely absent.

Behavior and social skills

NF1 patients commonly display difficulties in behavior and social skills. Parents and teachers predominantly report behavioral problems in the internalizing domain (overcontrolled behavior that contributes to distress to the child itself, such as anxiety, depression and withdrawal).[59, 67, 78-82] Some studies also reveal externalizing problems (undercontrolled behavior that contributes to distress to others, such as aggressive behavior), albeit to a lesser extent than internalizing problems.[59, 78, 80-82] About 40% of the NF1 patients meet diagnostic criteria for ADHD,[20, 78, 83] although only a small minority presents with the hyperactive subtype.[20] Children with NF1 and ADHD were found to respond well to methylphenidate treatment, which improved attention, behavior and social functioning.[83] NF1 has been associated with a higher incidence of autism (about 4%),[84] as well as other psychiatric and affective disorders, including dysthymia.[85]

Children with NF1 are frequently reported to have poor social skills,[78, 80, 81, 83] especially if they have co-morbid ADHD.[78] These difficulties are reflected in their interaction with other children, as children with NF1 are frequently picked on,[59] have problems with peers,[82] and have fewer friends than other children.[81] In addition, children with NF1 are considered by teachers and peers to be more sensitive and isolated, less likely to be leaders than other children,[81] and to be less independent than children without NF1.[59, 86] They do, however, seem to display equal, or even more pro-social behavior, such as being polite and helpful to others, compared to other children.[81, 82]

Interestingly, despite all problems mentioned above, children with NF1 themselves report a positive overall self concept,[78] rate their own social skills as normal or above average,[78] and have an above average academic self-concept compared to objective norms.[87] In addition, they do not confirm the reports of teachers and peers on sensitivity and isolation, or leadership qualities.[81]

Obviously, the effect of NF1 on the experience of daily life is not straightforward. Barton et al. proposed that children with NF1 could have unrealistic positive self-perceptions, which resembles the 'positive illusory bias' observed in otherwise healthy children with learning disabilities or ADHD.[87-89] Possibly, this bias arises from a self-protective mechanism to prevent confrontation with problems, or could reflect a focus on positive feedback only, or deficits in processing feedback to one's own behavior.[87] In NF1, the latter could possibly be related to NF1-specific neuropsychological dysfunction, such as their difficulty in interpreting social cues,[90] which could be secondary to problems in visual perceptual skills.

Quality of life

As can be expected from the numerous physical and cognitive problems associated with NF1, adult patients report a below average quality of life (QOL) across multiple domains of skin disease-specific and general health-related QOL questionnaires.[91, 92] These problems include emotional distress, inhibitions in physical and social functioning, and physical complaints. In many domains these scores correlate to disease visibility, disease severity or both.[91, 92] Parents of toddlers with NF1 indicate that their children experience problems with growth and development, physical functioning and behavior. In addition, parents themselves experience an impact of their toddler's NF1 on their personal time and emotions, and consider their child's health to be below average.[93] Another study revealed parents perceive problems in their child's motor, cognitive, social and emotional functioning.[79] The latter study is the only study so far that has also investigated QOL self-reports of NF1 children, revealing that children experience problems in the same areas as indicated by their parents, as well as in the domain of autonomy (independence).[79] One could imagine that the numerous cognitive and behavioral problems have a substantial impact on QOL scores of NF1 children. However, the relationship between cognitive and behavioral problems, and QOL, has not been investigated yet.

Motor performance

Children with NF1 frequently display problems with fine and gross motor functioning, including problems with fine motor speed, manual dexterity, balance and gait.[20, 53, 54, 56-58, 60, 68] Children show a delay in reaching motor milestones,[68] and are often described as being clumsy.[17, 59] One small study among 10 NF1 patients suggested impairments in the latencies and directions of saccadic eye movements.[94]

Unidentified Bright Objects

The most frequent NF1-related brain abnormalities are hyperintensities visible on T2 weighed Magnetic Resonance (MR) or FLAIR (Fluid Attenuated Inversion Recovery) images, so-called Unidentified Bright Objects (UBOs, see figure 3). These UBOs are found in about 70% of NF1 children, but tend to disappear in adulthood.[61, 95] UBOs are predominantly found in the globus pallidum, thalamus, cerebellum, brain stem and subcortical white matter.[61] The differentiation between UBOs and malignancies can be difficult. However, UBOs are not visible on CT or T1 weighed MR images, exert no mass effect, are not surrounded by edema, are not associated with focal neurological deficits, and do not enhance with gadolineum contrast. Previous studies showed that UBOs are not static and can disappear over time.[96-98]

The exact nature of UBOs is unclear. One study performed a post-mortem histopathological examination of brain areas that were diagnosed as UBOs on MRI, and reported spongiform myelinopathy with vacuolar changes.[99] These findings are confirmed by studies using Diffusion Weighted Imaging. These studies report higher Apparent Diffusion Coefficients (ADC-values) in NF1 brains at UBO-positive and UBO-negative sites compared to controls, suggesting an increased water content in UBOs and normal appearing brain of NF1 patients.[98, 100-102] However, the localization of this increased water content is still unclear.

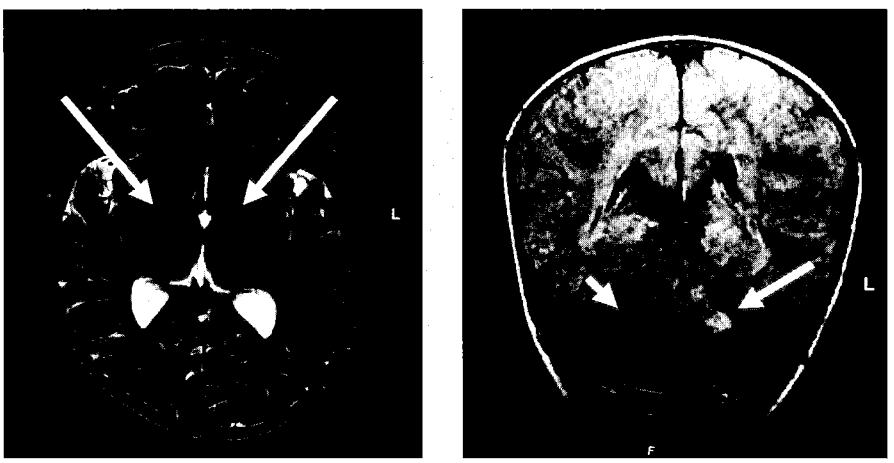

Figure 3: Unidentified Bright Objects (UBOs).

Left: Transversal T2 weighed MR (Magnetic Resonance) image showing bilateral UBOs in the basal ganglia (arrows) in a 9-year old NF1 patient *Right:* Coronal FLAIR (Fluid Attenuated Inversion Recovery) image showing a large UBO in the left cerebellar hemisphere (long arrow) and a smaller one in the right cerebellar hemisphere (short arrow) of a 2-year old NF1 patient.

Clinical correlates of cognitive problems in NF1

Genotype – phenotype relationships

It would be very useful if the cognitive abilities and disease severity of NF1 patients could be predicted from their specific type of NF1 mutation. Unfortunately, studies aimed at finding genotype-phenotype relationships are hampered by the broad mutational spectrum, and have not been succesful.[103-106] Only two strong relationships have been reported so far. Microdeletions of the NF1 gene are associated with a more severe clinical and cognitive phenotype,[107] and a 3bp in-frame deletion (c.2970-2972 delAAT) is reported to result in a

markedly mild clinical phenotype with possibly also a low frequency of learning disabilities.[108] The more severe phenotype of microdeletion patients is possibly mediated by the deletion of other genes in the microdeletion region. One of these, the RNF135 gene, was recently postulated as a candidate gene for the overgrowth, facial dysmorphism and possibly the more severe learning disabilities of NF1 microdeletic patients.[109] The large variation in phenotypes, even between individuals with identical mutations, suggests an important contribution of modifier genes.[110]

Influence of NF1-related brain abnormalities on cognition

The presence of brain tumors in NF1 does not seem to be related to lower cognitive functioning, unless children received radiotherapy, which has a strong negative impact.[74] There is no consensus on the relationship between UBOs and cognitive impairments in NF1. Some investigations reveal a connection between the presence, number, or localization of UBOs and cognition,[54, 56, 58, 60, 70, 111, 112] motor performance,[60, 111] and even between childhood UBOs and adult cognitive performance.[113] However, others find no relationship.[114, 115] Not all studies seem sufficiently powered to justify conclusions. Importantly, heterozygous *Nf1* mice show impairments in learning and memory but do not display UBOs or other gross brain abnormalities, at least not on a 4.7 Tesla MRI, indicating that the cognitive deficits in NF1 are not necessarily related to gross anatomical changes.[1, 116]

Molecular and cellular mechanisms underlying cognitive deficits

Animal models have been of great help to delineate the molecular and cellular mechanisms underlying cognitive deficits in NF1.

The etiology of cognitive deficits – lessons from *Nf1* mice

Nf1 heterozygous knockout mice display deficits in hippocampal-dependant learning and memory, and attention.[1, 7, 117] In addition, these mice show impaired hippocampal Long Term Potentiation (LTP),[1, 118] which is an *in vitro* measure of synaptic plasticity, the process of strengthening and weakening of neuronal contacts thought to be the neuronal substrate of learning and memory.[119] This deficit in LTP is observed when using a Theta Burst Stimulation (TBS) protocol, but not when using a High Frequency stimulation protocol, which may hint to increased sensitivity to GABA-agric inhibition. Indeed, GABA-agric inhibition was found to be increased in *Nf1* mice, and the GABA-A receptor antagonist picrotoxin can reverse the deficits in LTP.[1]

Importantly, the deficits in GABA-mediated inhibition, synaptic plasticity and learning in *Nf1* mice can be rescued by genetically reducing the level of N-RAS or K-RAS, suggesting that the cognitive phenotype of *Nf1* mice is ultimately caused by enhanced RAS signaling.[1] Theoretically, RAS activity can increase GABA (Gamma-aminobutyric acid)-mediated inhibition via postsynaptic changes (for instance by regulating GABA-A receptor dynamics), or via presynaptic changes (for instance by inducing the release of neurotransmitter vesicles containing GABA). A presynaptic mechanism seems plausible in NF1, as studies using mutant mice expressing the active H-Ras(G12V) gene revealed that active RAS facilitates neurotransmitter release, through inducing ERK-mediated phosphorylation of Synapsin 1, a presynaptic protein that is involved in neurotransmitter vesicle distribution.[7] Therefore, the most plausible model is that the cognitive phenotype of *Nf1* mice results from increased RAS/ERK/Synapsin-I signaling, leading to increased GABA release from inhibitory neurons, and impaired LTP (figure 4, left panel).

The Achilles' heel of RAS is that its activity is critically dependent upon its association to membranes, for which it requires post-translational isoprenylation (i.e. addition of a farnesyl or geranylgeranyl anchor).[120] Thus, pharmacological reduction of RAS activity with Farnesyl transferase inhibitors (FTI's) was found to restore the cognitive phenotype of *Nf1* mice.[1] These preclinical results offered perspectives at a drug therapy for cognitive impairments in humans, as they indicated that the cognitive deficits in NF1 are due to reversible changes in synaptic plasticity rather than structural anatomical abnormalities. However, since FTI's show significant side effects, a drug needed to be found that had the same effect but was also safe enough for long-term treatment of patients.

Statins decrease the synthesis of cholesterol, and isoprenoids (i.e. farnesyl and geranylgeranyl) by inhibiting HMG-CoA reductase, the rate-limiting enzyme in the mevalonate synthesis pathway. A breakthrough in the pursuit of a treatment for cognitive deficits in NF1 patients was made when it was discovered that short-term treatment of *Nf1* mice with lovastatin can reduce their increased RAS activity, and thereby rescue their impairments in synaptic plasticity, learning and memory, and attention[7] (see figure 4, right panel).

Statins are used to treat hypercholesterolemia in millions of people worldwide, and have a favorable safety profile in adults and children.[121, 122] This preclinical proof of principle warranted the initiation of translational clinical trials to assess the effect of statins on cognitive functioning in NF1 patients, the first of which is described in chapter 7 of this thesis.

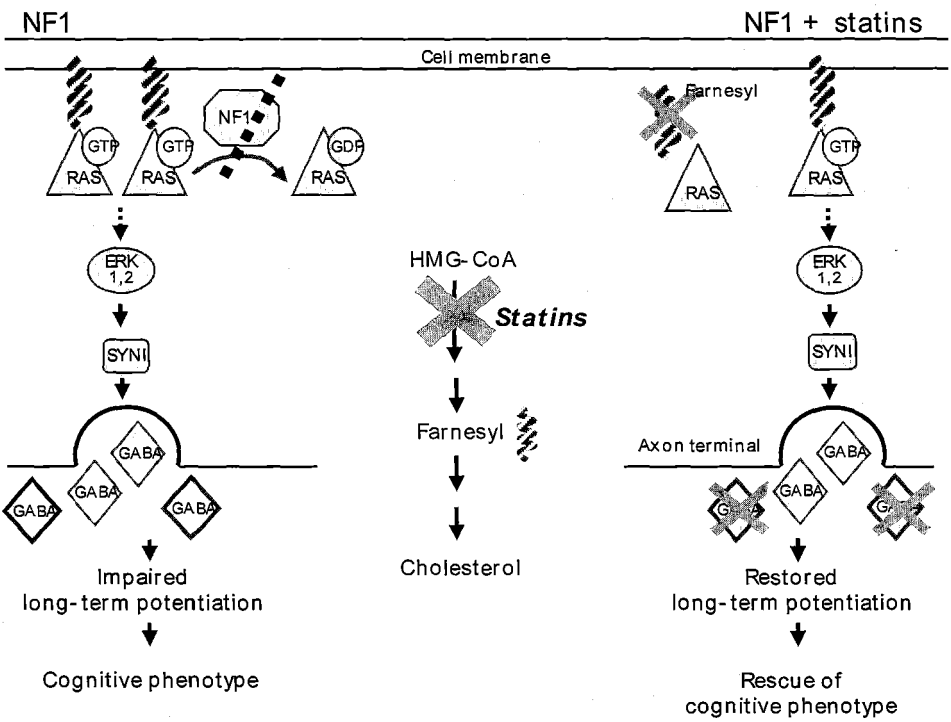

Figure 4: Simplified overview of the proposed presynaptic mechanism underlying the cognitive phenotype of *Nf1* mice and NF1 patients, and the proposed mechanism through in which statins restore the cognitive phenotype of *Nf1* mice. *Left:* Heterozygous loss of neurofibromin function leads to elevated activity of RAS, which, via increased ERK-mediated phosphorylation of synapsin 1, results in increased release of the inhibitory neurotransmitter GABA. This increased inhibition disturbs long-term potentiation, ultimately leading to the cognitive phenotype of NF1. *Middle:* Statins limit HMG-CoA reductase, thereby reducing the production of isoprenoids including farnesyl. *Right:* RAS activity is critically dependent upon isoprenylation. Reducing farnesyl availability with statins in *Nf1* mice normalizes the activity of RAS, which, via reduced ERK-mediated phosphorylation of synapsin 1, results in normalization of the release of the inhibitory neurotransmitter GABA. This restores long-term potentiation, ultimately rescuing the cognitive phenotype of *Nf1* mice.

The etiology of cognitive deficits – lessons from drosophila

Interestingly, *drosophila* flies with a homozygous neurofibromin deletion show impairments in immediate and long-term olfactory memory.[40, 123] The immediate memory deficit was shown to be related to decreased cAMP signalling,[40] whereas the long-term memory problems seems to be related to a loss of inhibition of RAS.[123] Strikingly, the cognitive phenotype of *Nf1* drosophila, like that of NF1 mice, can also be rescued with statin treatment.[124]

Because immediate memory is not tested in the *Nf1* mouse model the findings in drosophila leave open the possibility that not all cognitive deficits in NF1 are due to enhanced RAS

signaling. However, cDNA sequencing predicts drosophila neurofibromin has 60% amino acid identity to human neurofibromin,[39] whereas mRNA sequencing predicts mouse neurofibromin has over 98% amino acid conservation to human neurofibromin.[125] In addition, *Nf1* drosophila studied have a homozygous loss of neurofibromin, whereas *Nf1* mice still have one functional copy of the NF1 gene. Possibly, this explains why not all changes observed in *Nf1* flies can be found in *Nf1* mice.

The etiology of motor problems

A role for neurofibromin in motor functioning has been suggested by studies on mice with a heterozygous deletion of exon 23a, which show impaired performance on a motor task (the accelerating Rotarod test).[126] Possibly, the motor problems in NF1 originate from the cerebellum, as cerebellar Purkinje neurons are among the highest expressors of neurofibromin in the brain, [23, 127] and the cerebellum is one of the predominant sites for UBOs,[61] that have been related to motor problems.[60, 111] Although NF1 patients are not clearly ataxic, their clumsiness in movements could be related to deficits in the vermis, intermediate or lateral zones of the cerebellum.[128] The cerebellum plays an important role in motor performance, but also in motor learning, which refers to the ability to continuously adapt movements to optimize performance, a task which requires neuronal plasticity.[129-135] The motor learning capacities of children with NF1 have not been investigated so far.

Aims

Children with NF1 display a wide variety of cognitive problems, including neuropsychological deficits and problems with learning, behavior and motor performance. Preclinical studies indicate these deficits are reversible, and have also identified statins as candidate drugs for the treatment of cognitive deficits in NF1 patients.

The overall objectives of this thesis are to provide an overview of the impact of NF1 on daily life, to identify possible outcome measures that can be used to assess potential therapeutic interventions, and to investigate the effect of statins on cognitive problems in NF1 patients. These objectives are addressed in the following specific aims:

Aim 1

To review the current knowledge of the etiology of cognitive deficits in NF1 and related disorders within the Neuro-Cardio-Facial-Cutaneous and Hamartoma syndromes, and to review potential treatment options.

Aim 2

To provide insight into the impact of Neurofibromatosis type 1 on school performance.

Aim 3

To assess parent- and child perceived Health Related Quality of Life in children with NF1, and to identify potential targets for structural support.

Aim 4

To examine motor problems in children with NF1, and to investigate whether these problems arise from deficits in a specific brain area.

Aim 5

To explore the nature of T2-weighed hyperintensities on brain MR imaging in NF1 patients.

Aim 6

To assess the effect of simvastatin on neuropsychological, neurophysiological and neuroradiological outcome measures in children with NF1

Outline

The cognitive deficits of Neurofibromatosis type 1 (NF1) and several related disorders are discussed in the light of the shared underlying molecular and cellular disturbances of these diseases in **chapter 2.** The impact of NF1 on cognition and school performance, including need for remedial teaching and special education is discussed in **chapter 3.** Child- and parent perceived Quality of life, and potential determinants of reported problems are discussed in **chapter 4. Chapter 5** investigates whether impairments in motor functioning can be localized to functional abnormalities in specific brain areas, whereas in **chapter 6** focuses on the nature of T2 weighed hyperintensities observed on brain MR imaging in NF1 patients.

Chapter 7 reports the findings of a translational, randomized, double-blind, placebo-controlled trial to investigate the effect of simvastatin on cognitive functioning in children with NF1, using neuropsychological (**chapter 3**), neurophysiological (**chapter 5**) and neuroradiological (**chapter 6**) outcome measures.

Chapter 8 provides a discussion of the findings of this thesis, and a reflection on future research prospectives. The results of this thesis are summarized in **Chapter 9.**

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CHAPTER 2

Oncogenes on my mind: ERK and MTOR signaling in cognitive diseases

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Summary

Defects in rat sarcoma viral oncogene homolog (RAS)–extracellular signal regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (MTOR) signaling pathways have recently been shown to cause several genetic disorders classified as neuro–cardio-facial-cutaneous (NCFC) and Hamartoma syndromes. Although these pathways are well-known players in cell proliferation and cancer, their role in cognitive function is less appreciated. Here, we focus on the cognitive problems associated with mutations in the RAS–ERK and PI3K-MTOR signaling pathways and on the underlying mechanisms revealed by recent animal studies. Cancer drugs have been shown to reverse the cognitive deficits in mouse models of NCFC and Hamartoma syndromes, raising hopes for clinical trials.

Mutations in RAS signaling pathways are a leading cause for cognitive dysfunction

The RAS (rat sarcoma viral oncogene homolog) signaling pathways are evolutionary conserved pathways, transducing signals from membrane-bound receptors to proteins that regulate fundamental cell processes like cell growth and proliferation. Therefore, it is not surprising that genetic disorders with gain-of-function mutations in the RAS signaling pathways are characterized by benign and malignant overgrowths. This is a common phenotype for two groups of syndromes, classified in neuro-cardio-facial-cutaneous (NCFC) and Hamartoma syndromes. A high prevalence of mental retardation (see Glossary) and behavioral disturbances is also found among these patients (Table 1). Many of the genes associated with these diseases have been identified in the past few years and all are part of the ERK (extracellular signal regulated kinase) and MTOR (mammalian target of rapamycin) pathways (Figure 1 at the end of the chapter). For example, mutations in *SOS1* (Son of Sevenless, *Drosophila*, homolog 1) result in Noonan Syndrome, whereas mutations in *RAF-1* (v-raf-1 murine leukemia viral oncogene homolog 1) result in Noonan syndrome and LEOPARD, and mutations in *SPRED-1* (sprouty related EVH1 domain containing protein 1) in Neurofibromatosis type I-like syndrome.[1-11] Genetic alterations in RAS-ERK and PI3K (phosphoionositide 3-kinase)-MTOR signaling can be considered a leading cause of cognitive and behavioral impairments, collectively affecting ~ 1/1000 people.

Studies on rodents carrying mutations in components of the RAS-ERK signaling pathways indicate that postmitotic neurons have reprogrammed these signaling pathways to regulate synaptic plasticity (Table 2), believed to be the cellular basis for learning and memory (Figure 2 at the end of the chapter). Combining these neuroscience studies with molecular insights from cancer research has rapidly increased our understanding of the etiology of the cognitive deficits in the affected patients, and offers the opportunity to treat the cognitive deficits in NCFC and Hamartoma syndrome patients.

Cognitive deficits arising from genetic impairments in RAS-ERK signaling

The NCFC syndromes comprise a constellation of disorders that include Neurofibromatosis Type 1 (NF1), Noonan syndrome, Costello syndrome, Cardio-Facial-Cutaneous (CFC) syndrome, LEOPARD syndrome (an acronym for its cardinal features; lentigines, ECG conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness) and NF1-Like syndrome. All these syndromes are associated with some degree of mental impairment (Table 1). In general,

Table 1: Cognitive phenotypes of NCFC and Hamartoma syndromes[a]

Disease (prevalence)	Prominent phenotypical characteristics	Genes (% of cases associated with gene)[b]	Protein (function)	CNS features[a]		
				Very frequent (75- 100%)	Frequent (25-74%)	Less frequent (up to 25%)
Neuro-cardio-facial-cutaneous syndromes						
Neurofibromatosis type 1 (NF1, 1:3000)	Café-au-lait macules, skin fold freckling, Lisch nodules, cutaneous and plexiform neurofibromas[52]	*NF1* (95%) [49]	Neurofibromin (RAS-GAP) [a]	Low-average IQ,[15] specific deficits in attention, executive functioning and visual-spatial skills[13]	Learning disabilities, ADHD [a], social, emotional and behavioral problems, motor problems, speech problems, sleep disturbances, MRI [a] abnormalities, macrocephaly[13, 49]	Mild MR, autism, seizures, low grade gliomas[13, 47, 49]
Neurofibromatosis 1-like syndrome (rare)	Café-au-lait macules, skin-fold freckling, macrocephaly, lipomas; no neurofibromas or Lisch nodules[11]	*SPRED1*[11]	SPRED1 (inhibitor of Raf activation by RAS)			Frequency unknown. Some patients with macrocephaly, learning disabilities
Noonan syndrome (1:2000)	Typical facial features (hypertelorism, ptosis, low-set posteriorly rotated ears), webbed neck, short stature, cardiac problems [52]	*PTPN11* (50%)[1] *RAF1* (3-17%)[8, 9] *BRAF* (<2%)[9] *KRAS* (~2%)[4, 10, 62] *SOS1* (~9-13%)[6, 7] *MEK1* (~<2%)[63]	SHP2 (tyrosine phosphatase) RAF1 (serine/threonine kinase) BRAF (serine/threonine kinase) KRAS (small G-protein) SOS1 (GEF protein) [a] MEK1 (tyrosine/serine/ threonine kinase)	Low-average IQ[12]	Learning disabilities, motor problems, speech problems[69]	Mild MR, social and emotional problems, seizures[12, 69, 70]
LEOPARD (rare)[c]	Multiple lentigines, cardiac problems, short stature, Noonan-like facies, hearing loss[52]	*PTPN11* (>80%)[71] *RAF1* (~<7%)[8]	SHP2 (tyrosine phosphatase) RAF1 (serine/threonine kinase)			Mild MR[52]
Costello syndrome (rare)[d]	Coarse facial features, deep palmar/plantar creases, papillomata, short stature, cardiac problems [52]	*HRAS* (85-90%)[2, 72-74] *BRAF* (~4-6%)[63] *KRAS* (7%)[62] *MEK1* (~2-3%)[63]	HRAS (small G-protein) BRAF (serine/threonine kinase) KRAS (small G-protein) MEK1 (tyrosine/serine/ threonine kinase)	Mild to moderate mental MR [a], delay in language and motor development, macrocephaly[75, 76]	CNS abnormalities[76]	Irritability in young children, seizures[76]
Cardio Facial Cutaneous syndrome (CFC, rare)	"Noonan-like" with bitemporal constriction, sparse hair, ulerythaema ophryogenes, cardiac problems[52]	*BRAF* (43-78%)[3, 5, 77] *MEK1 (7-11%)*[5, 77] *MEK2 (6-7%)*[5, 77] *KRAS (5-8%)*[4, 77]	BRAF (serine/threonine kinase) MEK1 (tyrosine/serine/ threonine kinase) MEK2 (tyrosine/serine/ threonine kinase) KRAS (small G-protein)	Moderate to severe MR, hypotonia, marked delay in language and motor development[78]	Obsessive behavior, sleep disturbance, failure to thrive, macrocephaly, CNS abnormalities, seizures[78, 79]	Aggression[79]

Table 1 (continued)

Disease (prevalence)	Prominent phenotypical characteristics	Genes (% of cases associated with gene)[b]	Protein (function)	CNS features[a]		
				Very frequent (75- 100%)	Frequent (25-74%)	Less frequent (up to 25%)
Hamartoma syndromes[e]						
Tuberous Sclerosis Complex (TSC, 1:6000)	Hamartomas, hypo-melanotic macules, facial angiofibromas, renal angiomyolipomas	*TSC1* (19%)[80] *TSC2* (66%)[80 f]	Hamartin (binding partner of Tuberin) Tuberin (RHEB-GAP)	CNS abnormalities, seizures[81]	Bimodal IQ distribution: 50% normal IQ, 30% severe MR, specific deficits in attentional-executive skills, memory and language, psychiatric disturbances including autism[32]	Subependymal giant cell astrocytoma[81]
Bannayan-Riley-Ruvalcaba (BRR, rare)	Macrocephaly, hamartomas (lipomas, hemangiomas), penile macules[82]	*PTEN* (60%)[83]	PTEN (tyrosine phopshatase)	Macrocephaly, developmental delay[84]		Seizures[84]
Cowden Syndrome (rare)	Macrocephaly, Mucocutaneous lesions, high frequency of various types of malignancies[82]	*PTEN* (80-90%)[83]	PTEN (tyrosine phopshatase)		CNS abnormalities[85]	Learning disabilities, autism[82]

[a] Rare: up to a few hundred cases reported in literature; GAP: GTP-ase Activating Protein; GEF: Guanine nucleotide exchange factor; CNS: Central Nervous System; MR: mental retardation (mild: IQ 50-69; moderate: IQ 35-49; severe: IQ ≤ 34); ADHD: Attention Deficit Hyperactivity Disorder; MRI: Magnetic Resonance Imaging

[b] The ~ sign indicates that the mutation is reported in a subgroup of patients that is negative for a combination of other mutations associated with the disease, without specifying the size of the original population. In order to obtain an estimate of the prevalence of this mutation, we have corrected the percentage reported for the percentage in which these other mutations are postulated to occur, as reported in this table.

[c] LEOPARD is an acronym for the manifestations of this syndrome: multiple lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness.

[d] In the majority of studies, Costello patients with mutations in genes other than HRAS are re-diagnosed to CFC syndrome.

[e] The Hamartoma syndromes compromise Tuberous Sclerosis Complex, Peutz-Jeghers syndrome, and the subgroup of the PTEN-hamartoma tumor syndromes, consisting of Bannayan-Riley-Ruvalcaba, Cowden syndrome, Proteus syndrome and Lhermitte-Duclos disease. These latter group of diseases are all caused by germ line mutations in the PTEN gene.[83] However, because information on the cognitive phenotypes of Peutz-Jeghers syndrome, Proteus syndrome and Lhermitte-Duclos disease is very limited, these syndromes are not included.

[f] Calculated only for patients with a clinical diagnosis of TSC.

Noonan, LEOPARD, and NF1 are associated with mild cognitive deficits. However, despite low frequencies of mental retardation (IQ < 70) in NF1 and Noonan syndrome,[12-15] ~40% of the children require special education.[12, 13] In addition, even children with NF1 with a normal IQ can still display specific deficits in multiple cognitive domains, including visual-spatial skills, attention, executive functioning, and memory which puts them at risk for specific problems at school or work.[13, 14]

In contrast to these relatively mild phenotypes, patients with Costello or CFC syndrome present with high frequencies of mental retardation (Table 1). It is tempting to speculate that the generally milder cognitive phenotypes in Noonan, LEOPARD, NF1 and NF1-like syndrome are because of the fact that the causative mutations affect regulators of the RAS-ERK pathway. Mutations affecting the RAS, RAF and MEK (mitogen-activated and extracellular-signal regulated kinase kinase) proteins, found in Costello syndrome and CFC, might have a stronger effect on the output of the pathway (Figure 1). However, there are no data directly comparing activity levels of RAS-ERK signaling in brain tissue in the different disorders. Moreover, mutations in the same gene can yield variable phenotypes, such that patients with identical amino acid changes have been diagnosed with different syndromes (see Box 1 for striking examples). Thus, the relationship between genotype and cognitive phenotype is still poorly understood.

RAS-ERK signaling can modulate synaptic plasticity by regulating processes at both sides of the synapse: at the presynaptic side it modulates neurotransmitter release (in the axon terminal of the presynaptic neuron) and at the postsynaptic side, it controls protein synthesis (at the dendritic spines of the postsynaptic neuron) (Figure 1, 2).

A presynaptic RAS-ERK pathway modulates neurotransmitter release

By changing the amount of neurotransmitter released from its axon terminal, the presynaptic neuron can affect the strength of a synaptic connection (Figure 2). Several lines of evidence suggest that the RAS-ERK pathway is involved in this process, which is probably activated by binding of the neurotrophin BDNF (brain-derived neurotrophic factor) to the presynaptic TRKB (tyrosine receptor kinase type B) receptor. *Bdnf* mutant mice show a decrease in neurotransmitter release,[16] whereas stimulation of the RAS-ERK pathway by the application of BDNF, as well as by expression of the active *H-Ras(G12V)* (Harvey rat sarcoma viral oncogene homolog) gene, results in an ERK-dependent enhancement of neurotransmitter release. This is achieved by ERK phosphorylation of synapsin-I, a protein that binds to synaptic vesicles

containing neurotransmitters[16, 17] (Figure 1,2). The presynaptic RAS-ERK signaling pathway is not only controlled by neurotrophins. A recent study showed that also stress can induce presynaptic changes via the RAS-ERK pathway. Activation of this pathway is induced by corticosterone binding to the mineralocorticoid receptor,[18] but it is still unclear how activation of this receptor couples to RAS-ERK signaling.

BOX 1: WHAT'S IN A NAME? DIAGNOSING THE NEURO-CARDIO-FACIAL-CUTANEOUS SYNDROMES

A clinical or genetic diagnosis of a syndrome is invaluable to the affected patient and its parents in two aspects. First, they can identify themselves with families with the same disorder, and second, they expect to get a clear prognosis. However, the large overlap in phenotypes of the NCFC syndromes, the desire to diagnose patients at a young age, even though the phenotype might still be obscure, and the frequent lack of definite diagnostic criteria make it difficult to establish a diagnosis in an affected patient. This is especially true for patients with overlapping characteristics of Noonan, Costello and CFC syndromes.[10] Now that many genes have recently been identified, would a diagnosis based on the identified genetic mutation ensure a more accurate prognosis for the patient? Unfortunately, this is not the case, because even patients with identical mutations often have highly variable phenotypes. For example, identical mutations at D153V in *KRAS* were found in children diagnosed with Noonan syndrome,[62] severe Noonan with CFC features,[10] and CFC.[3] Likewise, mutations at E501K in *BRAF* are reported in patients with Noonan[9] and CFC,[3] and *BRAF* A246P mutations in CFC[3] and in Costello (the latter rediagnosed as CFC).[63] This indicates that modifier genes, of which none are identified at present, have an important role in shaping phenotypes in these syndromes.

Model organisms like mutant flies and mice are now used to identify these modifier genes. Therefore, future research might lead to a novel classification system based on a 'fingerprint' of a large number of selected genes that segregates patients on the basis of a certain prognosis (*eg.* malignancy risk or cognitive function) rather than on a mutated gene or a syndrome diagnosis.

Expression of the active *H-Ras(G12V)* gene in a subset of neurons that form stimulating synapses on their target neurons (excitatory neurons), resulted in enhanced synaptic plasticity and improved learning in an ERK-Synapsin-I-dependent manner.[17] This observation was surprising, because even though most of the NCFC disorders are also characterized by increased RAS signaling, the patients have learning deficits. The most probable explanation for this apparent paradox is that increased RAS-ERK-Synapsin-I signaling in these diseases is mostly restricted to inhibitory neurons. In contrast to excitatory neurons, inhibitory neurons form repressing contacts on their target neurons. Indeed, *Nf1* mice (*Nf1* heterozygous knock-out mice) show increased inhibitory transmission, which is probably mediated by enhanced release of the main inhibitory neurotransmitter in the central nervous system (GABA; Gamma-

Table 2: Mouse and rat mutants of genes associated with the RAS-ERK[a] and PI3K-MTOR signaling pathways and their phenotypes with respect to hippocampal function[b]

Gene	Mutation	Phenotype			
		Hippocampal-dependent Learning[c]	Synaptic plasticity[c]	Molecular signaling	Morphology
RAS-GRF1	*Ras-Grf1* homozygous knock-out mouse	Impairments in some spatial learning paradigms,[86] intact performance in others[22]	Impaired LTD,[23] intact LTP in some protocols[22] and slight impairment in others[23]	Intact NMDA-receptor induced ERK phosphorylation[23]	No apparent changes in brain morphology[22]
RAS-GRF2	*Ras-Grf2* inducible homozygous knock-out mouse[23]	Not known	Impaired LTP and decreased presynaptic plasticity	Decreased NMDA-receptor induced ERK phosphorylation	No apparent changes in brain morphology[87]
SynGAP	*Syngap* heterozygous knock-out mouse	Impaired spatial learning[88]	Impaired LTP[29, 88]	Increased basal ERK and MEK phosphorylation, increased NMDA-receptor induced ERK phosphorylation[88]	Increased number of AMPA-receptor clusters in neuronal cultures of homozygous knock-out mice[89]
HRAS	*H-Ras* homozygous knock-out mouse	Not known	Enhanced LTP in some protocols,[90] intact LTP in others;[88] increased NMDA-receptor mediated responses[90]	Increased phosphorylation of NR2A and NR2B subunits of the NMDA receptor;[90] intact basal ERK and MEK phosphorylation[88]	No apparent changes in brain morphology[88, 90]
HRAS	Forebrain and excitatory neuron-specific constitutively active *H-Ras* (*H-RasG12V*) mouse mutant[17]	Enhanced spatial learning	Enhanced LTP and increased presynaptic plasticity	Increased basal ERK and SYN1 phosphorylation, intact basal AKT phosphorylation	Increased amount of neurotransmitters ready for release (docked vesicles)
KRAS	*K-Ras* heterozygous knock-out mouse[19]	Impaired spatial learning	Impaired LTP	Not known	Not known
NRAS	*N-Ras* heterozygous knock-out mouse[19]	Intact spatial learning	Not known	Not known	Not known
NF1	*Nf1* heterozygous knock-out mouse (see Box 2)	Impaired spatial learning[19]	Impaired LTP and increased GABA-mediated inhibition[19]	Increased basal ERK and CREB phosphorylation;[50, 91] intact basal AKT phosphorylation[91]	Mild astrogliosis[92]
BRAF	Forebrain and excitatory neuron-specific *B-Raf* homozygous knock out mouse[93]	Impaired spatial learning	Impaired LTP	Intact basal ERK phosphorylation; decreased ERK phosphorylation after a learning paradigm	Not known
MEK1	Neuron-specific dominant-negative *Mek1* mutant[94]	Impaired spatial learning	Not known	Not known	No apparent changes in brain morphology
MEK1	Forebrain and excitatory neuron-specific dominant-negative *Mek1* mouse mutant[95]	Impaired long term spatial memory	Impaired late phase LTP	Decreased protein synthesis upon LTP inducing stimuli; decreased ERK, S6 and eIF4E phosphorylation upon LTP inducing stimuli and after a learning paradigm	Not known

Table 2 (continued)

Gene	Mutation	Phenotype			
		Hippocampal-dependent Learning[c]	Synaptic plasticity[c]	Molecular signaling	Morphology
ERK1	*Erk1* homozygous knock-out mouse	Enhanced spatial learning in some protocols,[96] intact performance in others[97]	Impaired LTP in some protocols,[96] intact LTP in others[96, 97]	Both increased[96] and intact[97] ERK2 signaling reported.	No apparent changes in brain morphology[96, 97]
ERK2	Knock-down mouse mutant with a 20-40% reduction in *Erk2* expression[98]	Impaired spatial learning	Not known	Not known	No apparent changes in brain morphology
PI3K	*p58α* (regulatory subunit of *Pi3k)* knock-out mouse[35]	Impaired spatial learning	Not known	Not known	Decreased synaptic density
PTEN	Mouse mutant with homozygous *Pten* deletion in limited neuronal populations (including hippocampus)	Impaired spatial learning[36]	Impaired basal transmission and LTP[37]	Increased basal AKT, MTOR and S6K phosphorylation[36]	Hypertrophy of cell soma, ectopic dendrites and axonal tracts and increased spine density[36, 37]
TSC1	*Tsc1* heterozygous knock-out mouse[38]	Impaired spatial learning	Not known	Not known	No neuronal abnormalities, no lesions by MRI
TSC2	*Tsc2* heterozygous knock-out rat	Intact spatial learning[99]	Impaired LTP and LTD and increased presynaptic plasticity[89]	Intact basal ERK phosphorylation, increased ERK phosphorylation upon LTP inducing stimuli	Adult animals are free of cerebral hamartomas, aged animals develop them at a slow rate[27]
TSC2	*Tsc2* heterozygous knock-out mouse[39]	Impaired spatial learning, was rescued by treatment with Rapamycin	Lower threshold for late phase LTP, was rescued by treatment with Rapamycin	Increased basal S6 phosphorylation, was rescued by treatment with Rapamycin	No apparent changes in brain morphology

[a] RAS, rat sarcoma viral oncogene homolog; ERK, extracellular signal regulated kinase; PI3K, phosphatidylinositol 3-kinase; MTOR, mammalian target of rapamycin; LTD, long-term depression; LTP, long-term potentiation; NMDA, N-methyl-D-aspartate; MEK, mitogen-activated and extracellular-signal regulated kinase kinase; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA, gamma-aminobutyric acid; CREB, cAMP response element-binding.

[b] Only rodent mutants in the direct RAS-ERK and PI3K-MTOR routes in which hippocampal function is specifically tested are presented.

[c] See Glossary.

aminobutyric acid). This increased inhibitory transmission seems to directly cause the impairments in plasticity and learning in these mutants[19] (Box 2). This is an interesting example of how a similar modification of the RAS-ERK pathway, can generate opposite systems-level outcomes by affecting two different types of neurons. However, it is not yet clear how the *NF1* mutation affects RAS-ERK signaling preferentially in inhibitory neurons.

BOX 2: TREATING COGNITIVE DEFECTS IN NF1 – LOST IN TRANSLATION?

Similar to NF1 patients, *Nf1* heterozygous knockout mice have problems in learning and attention.[19, 50] In addition, they show deficits in synaptic plasticity.[19] Importantly, these deficits can be rescued by genetically reducing the level of N-RAS or K-RAS, suggesting that learning and plasticity deficits in *Nf1* mice are caused by enhanced RAS signaling.[19]

RAS activity is critically dependent on its association to membranes, for which it requires the post-translational addition of a farnesyl or geranylgeranyl anchor. Statins decrease the synthesis of cholesterol, farnesyl and geranylgeranyl by inhibiting HMG-CoA (3-hydroxy-3-methylglutaryl-coenzyme A) reductase, the rate-limiting enzyme in the mevalonate synthesis pathway. Interestingly, treatment of *Nf1* mice or flies with statins cures their learning deficits.[50, 54] Statins are prescribed widely to treat hypercholesterolaemia, and have an excellent safety profile in adults and children. Therefore, the effect of simvastatin on cognitive functioning has recently been investigated in a randomized, placebo-controlled trial, involving 62 children with NF1.[64] Outcome measures included neuropsychological tests, MRI analysis and a neurophysiological test (measuring eye-hand movement control). Unfortunately, a three-month treatment resulted in a significant improvement in only one out of nine neuropsychological outcome measures, when compared to the placebo group. Several factors could have attributed to these disappointing results. First, it is conceivable that reversing deficits in higher cognitive functions in humans is far more difficult than reversing cognitive deficits in mice. This could be due to the greater complexity of the human brain. Second, the statin concentration that was reached in the human brain, could have been significant lower than in mice. This could be due to differences in metabolism, or to differences in blood-brain barrier permeability. Third, there was a large placebo or re-test effect, which brought 3 of the 9 neuropsychological outcome measures back to normal values. Since statins did not improve cognitive function in wild-type mice, it is possible that a ceiling effect was reached for these measures. Finally, it can not be excluded that the tests were not sensitive enough to capture a real improvement (see also Box 3). Because of all these biological and methodological issues, trials involving a longer treatment are now initiated. This would allow the brain more time to undergo changes, and would diminish the placebo and re-test effect by increasing the time in between testing moments. Moreover, a longer treatment would allow inclusion of real-life measures such as school performance.

The postsynaptic RAS-ERK pathway is an important signal integrator

Synaptic strength is not only controlled by regulating the amount of neurotransmitter release. In fact, most of the changes taking place during memory formation occur on the postsynaptic side

of the synapse. Influx of calcium ions through NMDA (N-methyl-D-aspartic acid) receptors is the pivotal trigger to initiate the process of synaptic strengthening, which can be measured *in vitro* (then referred to as LTP; long-term potentiation), and which is an absolute requirement for learning and memory. The RAS-GEFs (guanine nucleotide exchange factors) are recognized as major connectors between calcium ions and RAS-ERK activation, as they associate with NMDA receptors, and are activated by the influx of calcium ions through these receptors.[20, 21] Genetic studies suggest that RAS-GRF2 (guanine nucleotide-releasing factor) is the main GEF that drives RAS-ERK-dependent synaptic strengthening.[22, 23] However, an increase in calcium can also activate ERK through CaMKII (calcium/calmodulin-dependent protein kinase 2) mediated inactivation of SynGAP (synaptic RAS GTPase activating protein), a negative regulator of RAS signaling[24] (Figure 1). Besides calcium influx, the postsynaptic RAS-ERK pathway can also be activated by BDNF binding to the TRKB receptor, by the activation of β-adrenergic receptors and by a, more indirect, cAMP-PKA (protein kinase A)-dependent pathway[25-27] (Figure 1). Hence, the postsynaptic RAS-ERK pathway serves as a major signal integrator to control synaptic plasticity.

The postsynaptic RAS-ERK signaling pathway has many targets

How does the postsynaptic RAS-ERK pathway control postsynaptic plasticity? By changing the number of AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in the cell membrane, the postsynaptic neuron directly controls its sensitivity to glutamate and its probability to fire an action potential (Figure 2). Both over-expression of an active form of RAS in hippocampal neurons and silencing of SynGAP lead to an ERK-dependent increase in the amount of AMPA receptors in the postsynaptic membrane.[28, 29] This suggests a direct link between postsynaptic ERK signaling and AMPA receptor dynamics.

Protein synthesis is an absolute requirement to convert transient changes in synaptic strength into stable, long-lasting connections, and hence in stable memories. When measured *in vitro*, this phase of synaptic strengthening is referred to as late-phase LTP (L-LTP). ERK signaling plays a crucial role in controlling protein synthesis by regulating both transcription and translation events. One of the targets of the RAS-ERK pathway is the transcription factor CREB (cAMP response element-binding), which is important for memory formation.[26] The regulation of CREB seems to be sensitive to the application of BDNF[30] and its activation is dependent on several kinases downstream of ERK[26] (Figure 1). One of these, RSK2 (ribosomal S6 kinase 2),[26] is associated with the X-linked Coffin-Lowry syndrome (OMIM 303600) and patients with this disease present with mental retardation. Notably, the *CBP* (CREB-binding protein) gene, which

encodes an essential transcriptional co-activator of CREB, is mutated in Rubinstein-Taybi Syndrome, a disease characterized by severe mental retardation (OMIM 180849), again stressing the importance of proper ERK-dependent signaling in cognitive function.

Besides its roles in transcription, ERK signaling also controls translation in concert with the MTOR signaling pathway and more directly by activating the MNK (mitogen activated protein-interacting kinase) isoforms, which in turn activate eIF4E (eukaryotic initiation factor 4E)[31] (Figure 1). Collectively these studies indicate that RAS-ERK signaling plays a critical role in several major aspects of synaptic plasticity.

Cognitive deficits arising from genetic impairments in PI3K-MTOR signaling

Tuberous Sclerosis Complex (TSC) and the PTEN (phosphatase and tensin homolog)-hamartoma tumor syndromes, a group of clinical entities all resulting from germ-line mutations in *PTEN*, are classified as the Hamartoma syndromes (Table 1). The PTEN-hamartoma tumor syndromes present with mental impairments, however because of their rare nature detailed descriptions on the cognitive phenotypes are sparse. The cognitive profile of TSC patients is remarkably variable, with half of the patients having a normal IQ and about 30% an IQ below 20.[32] Similar to NF1, specific deficits in attention, executive functioning, memory and language are also common in TSC patients with a normal IQ.[32] The variation in cognitive abilities can in part be explained by differential effects of *TSC1* (Tuberous Sclerosis Complex 1 gene) versus *TSC2* (Tuberous Sclerosis Complex 2 gene) mutations (*TSC2* mutations tend to aggregate with more severe cases of mental retardation), and the abundance and localization of brain hamartomas and the presence and severity of epilepsy (Table 1).[32] The mutational spectrum of the two *TSC* genes is very broad, complicating research into a possible contribution of modifier genes to the variability in phenotype. Hence, no modifier genes that affect cognitive function have been identified. However, polymorphisms in the Interferon-γ gene[33] and in the gene encoding the DNA repair agent 8-oxoguanine glycosylase 1 (OGG1)[34] modulate susceptibility to renal angiomyolipomas in TSC patients, pointing to a role for modifier genes in the phenotypical variability of TSC.

PI3K-MTOR signaling controls protein translation

Rodent models have been developed for both TSC as for the PTEN-hamartoma tumor syndromes, and studies in these mutants reveal an essential role of PI3K-MTOR signaling in learning and memory[35-38] (Table 2). Both *Pten* homozygous knock-out mice and *Tsc1* and *Tsc2* heterozygous knock-out mice have impaired learning and show deficits in synaptic plasticity

(Table 2).[36-39] However, unlike the patients with PTEN-hamartoma tumor syndromes, *Pten* mouse mutants have severe disruptions in brain architecture,[37] which is probably related to these mice carrying a homozygous rather than a heterozygous deletion. It is not clear whether the learning deficits are secondary to these developmental brain abnormalities or the direct result of aberrant neuronal plasticity in the absence of PTEN. By contrast, a heterozygous *Tsc1* mouse mutant showed learning impairments in the absence of brain pathology or seizures, implying that the TSC proteins have a direct role in synaptic plasticity.[38]

Like the RAS-ERK pathway discussed above, the MTOR pathway is involved in the protein synthesis-dependent phase of synaptic strengthening. Rapamycin, a selective inhibitor of MTOR, specifically impairs this phase of synaptic strengthening and causes long-term memory deficits.[40, 41] Interestingly, all components of the PI3K-MTOR pathway and the complete translation machinery are present in dendrites,[41] suggesting that this pathway controls protein translation near the activated synapse (designated as 'local' protein translation). Indeed, BDNF stimulation is found to initiate MTOR-dependent protein translation in isolated dendrites.[42] Besides BDNF, activation of NMDA- and β-adrenergic receptors can also induce MTOR signaling.[25, 43] MTOR drives local protein translation through phosphorylation of its downstream targets, which include 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein) and S6K (S6 kinase)[44] (Figure 1). In addition to its important role as an initiator of local protein synthesis, MTOR can also suppress local translation of certain proteins, among which is the Kv1.1 potassium channel.[45]

As expected based on their increased MTOR signaling, *Tsc2* mutant mice show increased phosphorylation of S6 ribosomal protein, which is involved in protein translation (Figure 1). Consequently, a relatively weak stimulus is sufficient to recruit the protein synthesis-dependent phase of synaptic strengthening in these mutants. Paradoxically, this causes a learning deficit rather than a learning enhancement, probably because of inappropriate storage of unrelated or unprocessed information.[39]

It remains to be elucidated which dendritically targeted mRNAs are specifically regulated by MTOR, and how this couples to synaptic strengthening. However, a direct link has been established between PI3K signaling and AMPA receptor insertion, suggesting that this might be one of the main mechanisms by which MTOR signaling drives long lasting synaptic changes.[46] Taken together, these studies imply that a crucial balance of MTOR signaling is required to control neuronal protein translation, which is essential to long-term synaptic changes.

ERK and MTOR signaling in autism

Thus far, we have focused on the roles of ERK and MTOR signaling in cognitive function; however, behavioral problems are also commonly associated with both the NCFC and the Hamartoma syndromes. Evidence for a relationship with autism is somewhat limited for both NF1[47, 48] and Noonan syndrome[48], but autism is certainly a prominent characteristic of the Hamartoma syndromes (Table 1). One half of the TSC patients have autistic features [32]. Conversely, mutations in the *TSC* genes are found in 1% of autistic individuals and *PTEN* germ line mutations are found in as many as 17% of patients presenting with both autism and macrocephaly.[48] These are strikingly high percentages in light of the still obscure genetic knowledge on autism.

Mouse models for the PTEN-hamartoma tumor syndromes and TSC are found to recapitulate the social withdrawal phenotype as seen in autistic individuals.[36, 38] Taken together, the high incidence of autism in Hamartoma syndromes patients and the autistic phenotypes in mouse models for these syndromes make clear that aberrations in the PI3K-MTOR pathway can cause molecular and cellular changes that lead to autistic behavior. Thus, this pathway might be a major player in causing autism. However hampered by a lack of knowledge of the brain areas involved in autism, insight is limited into the exact mechanisms leading to autism upon enhanced PI3K-MTOR signaling.

Cognitive impairments are related to reversible changes in signaling rather than gross brain abnormalities

Structural brain abnormalities and seizures are part of the phenotypic spectrum of the NCFC and Hamartoma syndromes (Table 1). It could be argued that the cognitive deficits develop only secondary to these brain abnormalities. However, evidence for this idea is limited and contested. First, although infantile spasms are associated with a poor cognitive outcome in TSC,[32] clinical studies fail to show consistent data on a correlation between MRI abnormalities and cognition in TSC and NF1.[49] Second, cognitive impairments in most of the mouse models for NCFC and Hamartoma syndromes occur in the absence of structural brain abnormalities as seen in patients (Table 2). Third, as outlined in previous sections, the cognitive impairments found in these mouse models seem to arise from disturbances in the balance of neuronal signaling, because treatments with drugs specifically targeting these signaling disturbances can rescue both the cognitive deficits as the impairments in synaptic plasticity in mouse models for TSC and NF1 (Box 2).[39, 50] Interestingly, recent results show that, even though epilepsy correlates with poor cognitive outcome in TSC, this symptom can also be directly attributed to disturbed MTOR

signaling, and can be rescued with Rapamycin.[51] These findings suggest that the cognitive impairments are not caused by irreversible developmental abnormalities of the brain, but can be attributed to reversible changes in signaling.

Treating cognitive genetic disease – lessons from cancer

Cancer research has generated a wealth of knowledge on how to interfere with RAS-ERK and PI3K-MTOR signaling. By exploiting this knowledge we might be able to reverse the cognitive deficits associated with the NCFC and Hamartoma syndromes. However, there are several important aspects that have to be considered when using oncology drugs to treat cognitive deficits. First, animal studies suggest that both increased and decreased ERK or MTOR signaling result in cognitive impairments, indicating that a strict balance is required (Table 2). This is in marked contrast to tumors associated with these diseases, which are always caused by up-regulation of the ERK or MTOR pathway, and often require an additional second hit affecting the other allele (loss of heterozygosity) to become oncogenic. [52] Thus, treating cognitive deficits requires considerably more careful dosing than the treatment of cancer. Importantly, this implies that high doses of these drugs, as used in cancer treatment, might negatively affect cognitive function, which is of considerable concern (Box 3). Second, side-effects are acceptable in treating a life-threatening tumor, especially if the treatment is short. However, the treatment of cognitive deficits would probably be life-long and therefore requires an exceptionally good safety profile. Finally, many of the small molecule inhibitors used in cancer treatments are specifically designed to not be able to cross the blood-brain barrier, which makes them unsuitable to treat cognitive disorders.

Treatment of NCFC syndromes with inhibitors of the RAS-ERK pathway

Inhibiting RAS activity is a potential treatment mechanism for the cognitive impairments in the NCFC disorders (Figure 1). RAS activity can be diminished by attacking its Achilles' heel: its requirement to be post-translationally modified (Box 2). Both farnesyl transferase (FTase) inhibitors and statins can reduce RAS signaling in this manner, but although they show anti-proliferation effects *in vitro*, their success in treating cancer as a monotherapy has been limited (reviewed in [53]). Nevertheless, it is probable that the amount of RAS inhibition required to treat cognitive deficits is significantly lower than for tumor regression. Indeed, both FTase inhibitors and statins were sufficient to rescue cognitive and plasticity deficits of *Nf1* mice,[19, 50] and more recently to rescue learning impairments in *Nf1* mutant flies,[54] suggesting an evolutionary conserved mechanism. Despite these findings, a clinical trial assessing the effects of simvastatin in NF1 patients showed little effect (Box 2).

It has to be noted that the *in vitro* antiproliferative effects of both FTase inhibitors and statins cannot solely be ascribed to their ability to interfere with RAS signaling.[55] Therefore, we cannot rule out that the rescue of the learning deficits of *Nf1* mice is the result of other mechanisms. For instance, statins might reduce the synthesis of neurosteroids, because the rate-limiting step of steroid synthesis is the conversion of cholesterol to pregnenolone. Because neurosteroids directly activate the inhibitory GABA-A receptor,[56] reduction of neurosteroid levels might help to decrease the enhanced inhibition mediated by GABA-A signaling, as observed in *Nf1* mice.[19]

BOX 3: COGNITIVE FUNCTION AND CHEMOTHERAPY: THE CHEMOBRAIN

The increasing number of patients surviving cancer has aroused interest in how chemotherapy affects quality of life. Patients receiving conventional chemotherapy that causes DNA damage and cell death (e.g. platinum compounds) often report transient or even persistent cognitive impairments across various domains including working memory, executive function and processing speed (reviewed in ref. [65]). However, the precise impact of chemotherapy on brain function is a matter of debate for two major reasons. First, some of the studies reported a discrepancy between self-reported problems and objective neuropsychological tests, with no clear correlation between these two measures.[66, 67] Second, most studies are cross-sectional studies; hence, cognitive performance of the subjects before treatment is not known. Recently, several prospective (longitudinal) studies on this topic have been published, and although most studies suggest that chemotherapy has an impact on cognitive function, it does not seem as dramatic as reported by some earlier cross-sectional studies (for a review, see Ref [66]). Possibly, the effects in these prospective study designs were smaller, because patients are repeatedly assessed with similar tests, which can result in practice effects that might mask a real cognitive decline. However, one interesting aspect that was noted in several of these prospective studies was a greater than expected incidence of cognitive problems in these patients even *before* initiation of chemotherapy (see Ref [65] and references therein). Although several factors including psychological factors (e,g. stress, anxiety, depression after being diagnosed with a life-threatening disease) and biological factors (e.g. cytokine elevation) could cause this pretreatment deficit in cognitive functioning, it is tempting to speculate that certain polymorphisms in the genes the function in the RAS-ERK or PI3K-MTOR pathways result in increased cancer susceptibility as well as decreased cognitive function.

Because of the DNA-damaging nature of conventional chemotherapies, neuronal cell death is more likely to be an important mechanism underlying the induced cognitive problems than direct interference with synaptic plasticity. By contrast, the novel chemotherapies that are based on small molecule inhibitors directed against proteins in the pathways discussed in this review can directly impede synaptic plasticity. Thus, provided that they can cross the blood-brain barrier, they might severely affect cognitive function. For instance, MEK and MTOR inhibitors are found to affect cognitive function in wild type mice.[40, 68] Therefore, substantial animal and clinical studies will be required to assess the short-term and long-term effects of these new cancer treatments on cognitive function.

The RAS-ERK pathway can also be targeted with several newly developed small molecule inhibitors of B-RAF (v-raf-1 murine leukemia viral oncogene homolog B1) and MEK. Cancer trials with these inhibitors are underway (reviewed in [57]). However, this first generation of small molecule inhibitors is probably not suitable for treating cognitive deficits, because of low blood-brain permeability and significant side effects.

Treatment of Hamartoma syndromes with inhibitors of the MTOR pathway

The MTOR inhibitor Rapamycin (Sirolimus), applied as immunosuppressant in organ transplant patients, has already been successfully used to treat astrocytomas and angiomyolipomas in TSC patients.[33, 58, 59] Rapamycin is also shown to have anti-proliferative effects in patients with brain tumors caused by reduced PTEN activity.[60] A recent study revealed that rapamycin can reverse the cognitive deficits and aberrations in synaptic plasticity in *Tsc2* mutant mice.[39] In addition, a clinical trial to measure the effect of rapamycin treatment on renal hamartomas in TSC patients in conjunction with cognitive function (memory and executive skills) as secondary outcome measure is underway (NCT00490789; http://clinicaltrials.gov). Ideally, all future Rapamycin trials in TSC patients should include some measures to assess cognitive improvements and quality of life. The success of the cognitive improvements (if any) should then be carefully weighed against the drawbacks associated with a long-term treatment with rapamycin.

Finally, like RAS, the TSC target protein RHEB (RAS homolog enriched in brain) is crucially dependent upon farnesylation. This suggests that FTase inhibitors and statins might also help to treat the cognitive deficits in the Hamartoma syndromes.[61]

Concluding remarks

Here, we have emphasized the importance of the oncogenic RAS-ERK and PI3K-MTOR signaling pathways in cognitive functioning by focusing on the cognitive deficits associated with the NCFC and Hamartoma syndromes. There is a strong connection between genetic alterations in components of these pathways and cognitive dysfunction. Because of pioneering studies in cancer research, these signaling routes are very well characterized and rapid progress has now been made to understand their role in neuronal function. Animal studies revealed that the neuronal RAS-ERK and PI3K-MTOR pathways modulate neurotransmitter release, control synthesis of proteins required for stabilizing synaptic changes, and regulate receptor properties and dynamics. These processes play a pivotal role in synaptic plasticity, required for proper cognitive function. Recent targeted treatments in animal models of NCFC and Hamartoma

syndromes, using drugs designed for cancer treatment have been successful, and will undoubtedly stimulate the initiation of many clinical trials.

BOX 4 AREAS FOR FUTURE RESEARCH

- Investigating to what extent the mechanisms underlying the more severe neuro-cardio-facial-cutaneous (NCFC) disorders are different from the mechanisms underlying the NCFC disorders with only mild cognitive deficits.
- Investigating how certain mutations only affect a subclass of neurons (eg. Neurofibromatosis type 1 affects predominantly inhibitory neurons).
- Identification of the mRNAs whose translation is controlled by mammalian target of rapamycin (MTOR) and defining which are crucial for causing the cognitive deficits in the Hamartoma syndromes.
- Do the treatments, which can rescue cognitive functioning in mouse models of the Hamartoma syndromes, also rescue their autistic phenotypes?
- Compelling proof that the treatments that can rescue the cognitive deficits in mutant mice are also effective and safe in patients.
- Identification of small-molecule inhibitors of the extracellular signal regulated kinase (ERK) and MTOR pathways with minimal side-effects and that can cross the blood-brain barrier efficiently so that they can be used to treat cognitive deficits.

GLOSSARY

AMPA RECEPTOR: Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Subtype of the glutamate receptor, mediates fast excitatory synaptic transmission in the central nervous system.

ANGIOMYOLIPOMA: Benign kidney tumor composed of an abnormal collection of blood vessels *(angio)*, smooth muscle *(myo)*, and fat *(lipoma)*. Found in 70-80% of TSC patients.

ASTROCYTOMA: Benign brain tumor composed of undifferentiated, dysfunctional glial cells. Found in 10-20% of TSC patients.

AUTISM: A developmental disorder characterized by a triad of symptoms: a qualitative impairment in social interaction, qualitative impairments in communication, and restricted, repetitive and stereotyped patterns of behavior.

DENDRITE: A neuronal process arising from the cell body that receives synaptic input. From the viewpoint of a specific synapse, this dendrite lies on the postsynaptic side.

EXCITATORY NEURON: A neuron that forms stimulatory contacts on its target neurons, and thereby increases their probability to fire. Glutamate is the most common neurotransmitter released by excitatory neurons.

GLOSSARY (CONTINUED)

GABA: Gamma-aminobutyric acid; Most abundant inhibitory neurotransmitter in the central nervous system.

GLUTAMATE: Most abundant excitatory neurotransmitter in the central nervous system.

HAMARTOMA: A benign tumor-like growth consisting of a disorganized mixture of cells and tissues normally found in the area of the body where the growth occurs.

HIPPOCAMPUS: Part of the brain essential for memory formation. In rodents its function is typically assessed with maze-tasks.

INHIBITORY NEURONS: A neuron that forms inhibiting contacts on its target neurons, and thereby reduces their probability to fire. In the central nervous system, GABA is the most common neurotransmitter released by inhibitory neurons.

LTD/LTP: Long-term depression/Long-term potentiation; An *in vitro* measure of synaptic weakening and strengthening, respectively. LTP can be subdivided in an early phase, (1-2 hours after LTP induction) requiring posttranslational changes only, and a late phase, which requires the synthesis of new proteins. The protein synthesis-dependent phase of synaptic strengthening is required for long term memory formation.

MENTAL RETARDATION (MR): The combination of an IQ <70 (normal IQ is 100 ± 15) with significant limitations in at least two areas of adaptive behavior (e.g. communication, daily living skills or social skills), apparent before the age of 18. An IQ of 69–50 is defined as mild; 35–49 as moderate; 20–34 as severe, and <20 as profound MR.

NEUROTROPHINS: Family of proteins, which are important for neuronal survival in the developing brain, and play a role in synaptic plasticity in the mature brain.

NMDA RECEPTOR: A subtype of glutamate receptor. Mediates calcium influx during LTP induction.

PLASTICITY (SYNAPTIC/NEURONAL): The ability of neurons to change the strength of synaptic contacts or their excitability. These processes are required for memory formation.

POSTSYNAPTIC: The side of the synapse on the dendrite where the receptors are located which are receptive to the released neurotransmitter molecules.

PRESYNAPTIC: The side of synapse on the axon terminal where neurotransmitter molecules are released, which convey signals to the target cell.

FIGURE 1: OVERVIEW OF NEURONAL RAT SARCOMA VIRAL ONCOGENE HOMOLOG (RAS)- EXTRACELLULAR SIGNAL REGULATED KINASE (ERK) AND PHOSPHATIDYLINOSITOL 3-KINASE (PI3K)- MAMMALIAN TARGET OF RAPAMYCIN (MTOR) SIGNALING PATHWAYS AND THE ASSOCIATED NCFC AND HAMARTOMA SYNDROMES

The RAS-ERK and PI3K-MTOR pathways are both regulated by the activity of small GTP-binding proteins (G-proteins), RAS and RHEB, respectively (accentuated in the figure with a thick border). These small G-proteins can reside in two different states: a GTP (guanosine triphosphate)-bound active state and a GDP (guanosine diphosphate)-bound inactive state. Their activity level is determined by interactions with GAP (GTPase activating protein) and GEF (Guanine exchange factor) proteins. The different GEF proteins promote the exchange of GDP for GTP, leading to enhanced activity, while GAP proteins catalyze the hydrolysis of GTP to GDP, leading to suppression of activity.

Activation of RAS is initiated by calcium influx through N-methyl-D-aspartate (NMDA) receptors, which in turn activates RAS-guanine nucleotide-releasing factor (RAS-GRF2) and inactivates synaptic RAS GTPase activating protein (SynGAP).[20-24, 26, 29] RAS can also be activated after mineralocorticoid receptor (MR) activation by corticosterone (cort), β-adrenergic receptor (β-AR) activation by noradrenaline (NA) or by brain-derived neurotropic factor (BDNF) binding to the tyrosine receptor kinase type B (TRKB) receptor, which initiates RAS signaling by activating the GEF protein: Son of Sevenless, *Drosophila*, homologue 1 (SOS1), a GEF-homolog.[16-18] Src homology protein 2 (SHP2) stimulates this activation in as yet undefined ways. Active RAS activates RAF, which induces a phosphorylation cascade ultimately leading to activation of ERK and its downstream targets. At the presynaptic side these include Synapsin-I (SynI), which modulates neurotransmitter release. At the postsynaptic side, ERK activates ribosomal S6 kinase 2 (RSK2) and myocardial Snf1 (sucrose nonfermenting 1)-like kinases 1, 2 (MSK 1, 2), which in turn activate the transcription factor cAMP response element-binding (CREB). Also, ERK activates mitogen activated protein-interacting kinases 1, 2 (MNK1, 2), which signal for translation. Also, ERK directly modulates the dynamics of ion channels including the potassium channel Kv4.2. All these processes all influence the strength of the synapse, essential to learning and memory formation. MTOR is a major controller of dendritic translation through its downstream targets S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). MTOR is driven by RHEB activity, which is increased by inhibition of Tuberin-GAP (TSC2) activity. This could occur directly by RAS-PI3K signaling or indirectly through RAS-ERK signaling. Dashed lines represent interactions of which not all details are elucidated at present.

The genes mutated in the different syndromes are depicted in the dark boxes, with the name of the syndrome(s) next to them. The genes shown in the light boxes are not (yet) associated with a syndrome, but are plausible candidate genes for cases in which no mutation is identified. The plus and minus signs indicate whether the identified mutations up- or down-regulate ERK or MTOR signaling (based on in *vitro assays*). Note that the vast majority of the mutations encountered in the NCFC and Hamartoma syndromes lead to enhanced ERK or MTOR signaling.

Abbreviations: SPRED1: sprouty related EVH1 domain containing protein 1, PIP3: Phosphatidylinositol (3,4,5)-trisphosphate, PIP2: phosphatidylinositol (4,5)-bisphosphate, PDK1: Pyruvate dehydrogenase kinase, isozyme 1, eEF2-K: eukaryotic elongation factor 2 kinase AKT: v-akt murine thymoma viral oncogene homolog, S6K: S6 kinase, eIF4B/E: eukaryotic translation initiation factor 4 B/E, eEF2-K: eukaryotic elongation factor-2 kinase.

Phos pho lipid
Isoprenyl anchor
kinase
GAP protein
GEF protein
Ion channel
small G-protein
agonist
Effector/ other
Phospha tase
Receptor
glut
Ca2+
NMDAR
cort
NA
BDNF
MR
ß AR
TRKB
Cell membrane
SynGAP
GDP
RAS
RAS GRF2
SHP2 (PTPN11)
Noonan, LEOPARD
NF1
GTP
SOS1
Noonan
PI3K
PIP2
PTEN
PIP3
PTEN- hamartoma tumor syndromes
PDK1
AKT
NF1– like Syndrome
SPRED1
Noonan, CFC, Costello
RAF1
BRAF
MEK1,2
ERK 1,2
TSC1/2
RHEB
RHEB GEF
mTOR
Tuberous Sclerosis Complex
Presynaptic side
Synl
Neurotransmitter release
Postsynaptic side
MSK 1,2
Coffin Lowry
Rubinstein Taybi
CREB
Transcription
Kv4.2
Ion channel modulation
MNK 1,2
4E-BP1
S6Ks
eIF4E
S6, eIF4B, eEF2-K
Translation

Figure 1

FIGURE 2: THE OUTPUT OF ERK AND MTOR SIGNALING IN MITOTIC CELLS AND NEURONS

(a) In non-neuronal, mitotic cells, extracellular signals such as growth factors and cytokines induce proliferation, differentiation and cell cycle progression via activation of ERK and MTOR pathways. However, neurons (b) are mostly post-mitotic, and the ERK and MTOR pathways are recruited for a process called synaptic plasticity, important in memory formation. Synaptic strength of a synapse is strongly dependent on the amount of neurotransmitter (glutamate) released presynaptically and on the amount of glutamate-responsive a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors present in the postsynaptic cell membrane. On different stimuli, including calcium influx via the N-methyl-D-aspartate (NMDA) receptor and brain-derived neurotropic factor (BDNF) binding to pre- and postsynaptic TRKB receptors, ERK and MTOR pathways change synaptic strength by both modulating neurotransmitter (glutamate) release and by regulating the insertion of AMPA receptors, which are activated by glutamate.

Abbreviations: glut: glutamate, G-protein: guanine nucleotide binding protein, GRF: growth factor, GTP: guanosine triphosphate, TRF: transcription factor.

Ion channel
Receptor
Agonist
small G-protein
kinase
Transcription factors
GRF
GRF Receptor
GTP
RAS
mTOR
ERK 1,2
Translation
Transcription
Transcription factors
Proliferation, Differentiation, Cell survival
A) Non-neuronal cell
Presynaptic side
ERK 1,2
Neurotransmitter release
RAS
GTP
TRKB
glut
glut
Synaptic plasticity
BDNF
BDNF
glut
glut
TRKB
NMDAR
AMPAr
Insertion AMPA receptors
GTP
RAS
mTOR
Translation
ERK 1,2
Postsynaptic side
TRF
Transcription
B) Excitatory neuron

Figure 2

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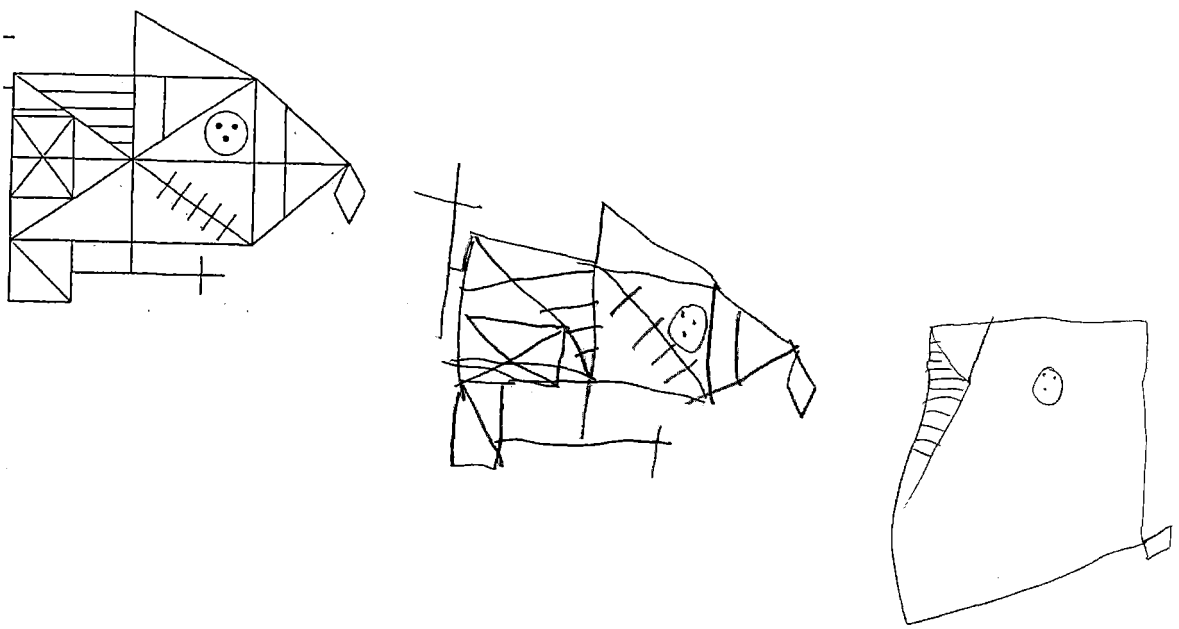

Example of the Rey Complex Figure Test (left), with the copy (middle) and delayed recall (drawn by heart after 15 minutes; right) versions drawn by a 10-year old girl with NF1.

CHAPTER 3

Impact of Neurofibromatosis type 1 on school performance

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Abstract

School functioning of 86 Dutch Neurofibromatosis type 1 children (7-17 years) was analyzed using teacher questionnaires to determine the impact of Neurofibromatosis type 1 on school performance. In all, 75% of the Neurofibromatosis type 1 children performed more than one standard deviation below grade peers in at least one of the domains of spelling, mathematics, technical reading or comprehensive reading. Furthermore, Neurofibromatosis type 1 children had a 4-fold increased risk for attending special education, and a 6-fold increased risk for receiving remedial teaching for learning, behavior, speech and/or motor problems. Children without any apparent learning disability still frequently displayed neuropsychological deficits. Only 10% of the children did not show any school-functioning problems. Finally, it was found that the clinical severity of Neurofibromatosis type 1 correlated with the cognitive deficits. Taken together, we show that Neurofibromatosis type 1 has profound impact on school performance. Awareness of these problems may facilitate timely recognition and appropriate support.

Keywords:
Neurofibromatosis Type 1 / learning disabilities / children

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease with an incidence of 1 in 2500 to 3000 individuals, of whom 50% are *de novo* cases.[1] Characteristics of NF1 are various neurocutaneous manifestations, including café au lait maculae, axillary freckling, neurofibromas, and Lisch nodules. Minor disease characteristics include developmental delay, poor motor skills and speech problems.[2] Clinical presentation of NF1 is highly variable even within families. In children, the most frequent complication of NF1 is a cognitive impairment.[3] Neuropsychological deficits include a lowered average intelligence quotient (IQ), problems with visual-spatial skills, memory, language, executive functioning, and attention (reviewed in [4]). In addition, children with NF1 have poor social skills, and up to 40% have attention-deficit hyperactivity disorder.[5, 6]

Until recently, little attention was paid to the impact of NF1 on school performance. Estimates for the occurrence of learning disabilities vary from 30 to 70%.[6-8] This large variation is likely due to different definitions for learning disabilities, selection bias of the study groups, or small sample size (reviewed in [4]). Importantly, all previous studies on learning disabilities associated with NF1 have solely used academic achievement tests taken at a clinical setting. Although these measurements reliably reflect the academic achievement level of a child, they do not take into account how this level was achieved. Receiving intensive remedial teaching or special education may seriously confound the interpretation of academic achievement test scores, and this may result in a significant underestimation of the learning disabilities and school problems associated with NF1. Therefore, to get a more realistic assessment of school performance, it is important to combine these different types of information on school functioning. However, quantitative studies on the level of special education, remedial teaching, or grade repetition in these children are largely absent.

The aim of this study is to determine the impact of NF1 on school performance of Dutch children with NF1 by examining the learning disabilities displayed in the school setting and by determining the relative risk for receiving extra support in the form of grade repetition, remedial teaching, and special education. In addition, we investigated the relationship between the clinical severity of NF1 and the cognitive deficits.

Methods

Patients

Participants were recruited from the multidisciplinary pediatric NF1 outpatient clinic of the Erasmus MC – Sophia Children's Hospital, Rotterdam. This outpatient clinic is a supraregional reference center that predominantly receives patients from the southwestern part of the Netherlands (about 3 million citizens). Data for this study were obtained in the context of a larger ongoing study on NF1 and cognitive functioning. Inclusion criteria were: NF1 diagnosis according to the criteria of the National Institutes of Health,[9] 7 to 17 years of age for the school performance questionnaire, 8 to 17 years of age for the neuropsychological assessment, and informed consent from parents and children aged older than 12 years. Exclusion criteria were: segmental NF1, pathology of the central nervous system (other than asymptomatic gliomas), deafness, severely impaired vision, use of anti-epileptics, inefficient production or comprehension of the Dutch language, and severe mental retardation (Full Scale IQ below 48, to exclude children with an IQ score below the range covered by the Wechsler Intelligence Scale for Children, Revised, Dutch version).

In all, 126 children fulfilled age criteria. Twelve children were excluded on the basis of segmental NF1 (n=3), use of anti-epileptics (n=3), pathology of the central nervous system (hydrocephalus, n=3), severe mental retardation (n=1) and inefficient production or comprehension of the Dutch language (n=2). The remaining 114 children were invited to participate in the school performance questionnaire and neuropsychological assessment.

Disease severity was scored by an experienced pediatrician of the NF1 team (A.G.B.) according to the Riccardi Scale,[10] modified to exclude cognitive aspects of NF1. Minimal NF1 was scored in the absence of features that compromise health (when only harmless clinical features such as *café au lait* maculae, freckling, and Lisch nodules were present). Mild NF1 was scored when minor medical complications, such as small stature or discrete plexiform neurofibroma, were present. Moderate NF1 was scored in case of complications that were a significant compromise to health, such as paravertebral neurofibromas or hypertension. Severe NF1 was scored in case of malignancy. Familial or sporadic NF1 was determined by the pediatrician from the family history. Informed consent was received from all participants. This study was approved by the medical ethical committee of the Erasmus MC – Sophia Children's Hospital.

School Performance

Teachers of the participating patients were requested to complete an abbreviated version of the Teacher's Report Form,[11] with additional quantitative and qualitative questions on remedial teaching. Teachers reported the most recent scores on technical reading, comprehensive reading, spelling, and mathematics from the Dutch Student Monitoring System. This system is a government-enforced system of standardized and validated academic performance tests for technical reading, comprehensive reading, spelling, and mathematics assessed 3 times a year in all grades of primary school in the Netherlands.[12] The tests most frequently used for technical reading, comprehensive reading, spelling, and mathematics are all rated sufficient to good on norms, good on reliability, and good on construct validity by the Committee on Test Affairs Netherlands. Key cutoff criterion for admission to a school for the mentally retarded in the Netherlands is IQ<60. Cutoff criteria for admission to a school for the learning disabled are 1) IQ 61-89, or 2) IQ>80 with a learning efficacy below 75%, or 3) IQ in the normal range in combination with severe visual, speech- language and hearing, motor, social-emotional, or behavioral problems.[13, 14]

Neuropsychological Assessment

Neuropsychological tests developed for children were administered to assess cognitive skills in six domains: 1. Intelligence (Wechsler Intelligence Scale for Children, Revised, Dutch version), 2. Memory (Rey Auditory Verbal Learning Test for verbal memory; Rey Complex Figure Test – delayed recall for nonverbal memory), 3. Language (Peabody Picture Vocabulary Test III for receptive language; Boston Naming Test for expressive language), 4. Visual-spatial skills (Judgment of Line Orientation task for line orientation; Rey Complex Figure Test – copy for visual integration; and Beery Developmental Test of Visual-Motor Integration for visual motor integration), 5. Executive skills (Trailmaking Test A and B for rote memory and divided attention; Animal naming for verbal fluency; Wisconsin Card Sorting Test for concept formation and perseverations), and 6. Attention (Stroop Color-Word Test for Selective attention; Cancellation Test – speed for sustained attention; Cancellation Test – attention fluctuations for attention fluctuations).[15, 16] All tests were administered in their Dutch versions and scored by a single pediatric neuropsychologist. To allow comparison across ages, neuropsychological scores were converted into Z-scores (deviation from the mean of a normal population). The evaluator was not informed on the medical status or school results of the patient. The neuropsychological testing took 3.5 hours to complete, and was divided into 2 sessions (2 hours and 1.5 hours) with a break of 1 hour in between.

Definitions

REMEDIAL TEACHING: Structural assistance on top of regular class assistance offered for problems with learning, motor function, speech, and/or behavior.

DIDACTIC SCORE: The test-specific score on one of the didactic tests used in the student monitoring system.

LEARNING EFFICACIES: To compare students across tests and ages, didactic scores are converted into learning efficacies. Hereto, the didactic score was first converted into a didactic age equivalent using normative conversion tables.[12] Learning efficacy was then calculated by dividing the determined didactic age equivalent by the actual months of education a child received in primary school (= didactic age). One school year consists of 10 didactic months. For example, if a child at the end of 5th grade (didactic age 5 x 10 = 50 months) has a didactic age equivalent on mathematics of 40 months, the learning efficacy of this child is 40/50 x 100%= 80%. Didactic age equivalents are required by the Dutch government for reporting progress of students.[17] The normative average score at a certain didactic age per definition equals a learning efficacy of 100% (didactic age = didactic age equivalent).

LEARNING DISABILITY: Learning disability was defined as a learning efficacy for technical reading, comprehensive reading, spelling, or mathematics of more than 1 standard deviation below the average (< 85%). A learning disability was termed specific if occurring with a normal IQ (IQ $\geq$ 85), and termed general with an IQ of more than 1 standard deviation below the mean (IQ < 85). To determine whether there was a specific or general learning disability, IQ was obtained from neuropsychological assessment or from the school performance questionnaire (question X of the Teacher's Report Form if the reported score was less than 1 year old and obtained with Wechsler Intelligence Scale for Children, version III or Revised, Dutch version). When learning efficacies for all four didactic domains were available, patients were assigned to one of the following groups: a No Learning Disabilities group, a Specific Learning Disabilities group (children with learning disabilities on one or more of the didactic domains but a normal IQ) or a General Learning Disabilities group (children with learning disabilities on one or more of the didactic domains and an IQ of more than 1 SD below the mean). If for a particular domain, scores were not present or if only qualitative instead of quantitative scores were available, these scores were treated as missing values in that specific domain.

Statistical Analysis

Data were analyzed in SPSS 12.0 (SPSS Inc, Chicago, Illinois) using a parametric test for continuous variables (2-sided independent t-test, Analysis of Variance [ANOVA] with a post-

hoc 2-sided t-test) and nonparametric tests for categorical variables or if n<20 (binomial test, chi-square test, Kruskal-Wallis test with post hoc Mann-Whitney test). The chi-square test was used to compare variance of our study group with that of the normal population. The Kolmogorov-Smirnov test was used to control for normal distribution.

Results

Informed consent for the school performance questionnaire was received for 89 children (response 78%). The questionnaire was completed by 86 of the 89 teachers (response 97%). School type was specified for all 86 children. Learning efficacies for one or more of the domains of technical reading, comprehensive reading, spelling, or mathematics and presence or absence of a learning disability could be calculated from quantitative scores of 75 children. These included 70 scores for technical reading, 61 for comprehensive reading, 69 for spelling, and 65 for mathematics. For 54 children, all 4 didactic scores were available. Remedial teaching was scored for 75 children and grade repetition for 70 children. Informed consent for the neuropsychological examination was received for 62 children (response 54%). Table 1 provides an overview of the patient characteristics.

Table 1: Patient characteristics

Characteristic	Number of patients (n=86)
Sex: Male/Female	47/39 (54.7/45.3%)
Age at assessment (years)	11.9 ± 2.5
Familial NF1/Sporadic NF1/unconfirmed	36/48/2
Modified Riccardi scale	
Scale I (minimal)	30
Scale II (mild)	30
Scale III (moderate)	25
Scale IV (severe)	1
Using medication for attention deficit-hyperactivity disorder	14

NF1: Neurofibromatosis type 1.

Learning Disabilities

School performance of children with NF1 was substantially impaired on all 4 domains of technical reading, comprehensive reading, spelling, and mathematics (table 2). Mean learning efficacies were significantly lower than normative grade-peer average (100%). Children with NF1 follow an average learning efficacy curve of 75% (at -1.7 SD from average, t=-7.66,

p<0.0005), which is at the cutoff level of admission to a school for the learning disabled. Learning disabilities (learning efficacy below 85% [<-1 SD]) were present in at least 56/75 (75%) of the children with NF1. Even when using a –2 SD cut-off (learning efficacy < 70%), 47/75 children (63%) displayed impairments in one or more didactic domains. Using IQ data of these children, we were able to determine whether a child had a specific learning disability for a given didactic domain (a learning efficacy on a specific didactic domain below 85% but with normal IQ) or a general learning disability (a learning efficacy on a specific didactic domain below 85% with IQ<85). This showed that specific and general learning disabilities were distributed equally over the academic areas (see table 2).

Table 2: Overview of the learning disabilities found in our patient group per didactic domain.

Didactic domain	Mean learning efficacy in % (SD)	Specific learning disability (%)	General Learning disability(%)
Spelling	70 (31)**	19/69 (28%)	26/69 (38%)
Technical Reading	74 (30)**	19/70 (27%)	25/70 (36%)
Mathematics	77 (35)**	15/65 (23%)	25/65 (39%)
Comprehensive Reading	78 (35)**	12/61 (20%)	22/61 (36%)

**: P-value <0.0005 compared to normative grade peer average (100%).

For 54 children, quantitative school performance data on all 4 didactic domains were present. This allowed us to assign them to a General Learning Disabilities group (learning disabilities on one or more of the didactic domains, and IQ<85), a Specific Learning Disabilities group (learning disabilities on one or more of the didactic domains, but IQ≥85) or to a No Learning Disabilities group (children without learning disabilities in any of the four didactic domains). On the basis of these criteria, 21 children (39%) were assigned to the General Learning Disabilities group, 21 children (39%) to the Specific Learning Disabilities group and only 12 children (22%) to the No Learning Disabilities group.

Remedial Teaching, Special Education and Grade Repetition

Special education was attended by 37% of the children, which is an odds ratio of 4.1 compared with the average population[18] (Table 3). In total, 33% attended a school for the learning disabled, and 5% attended a school for the mentally retarded. Forty percent of the children with NF1 repeated a grade in their school career. In primary school, this was significantly more frequent compared with the regular population (17% versus 1.9%, binomial test, p<0.0005).[19] The majority (85%) of the children with NF1 received remedial teaching for learning problems (didactical remedial teaching), fine and gross motor problems (motorical), speech problems (logopedical) and/or behavioral problems (behavioral), an odds ratio of 5.6 compared with 15%

in the Dutch population.[20] A combination of 2 or more types of remedial teaching was given to 52% of all children, mostly including remedial teaching for learning problems.

Nineteen out of 75 children had no learning disabilities. However, 12 (63%) of these children received remedial teaching, of which 5 (40%) specifically for learning problems. If we look at the children who were scored for the school type, grade repetition, remedial teaching, and learning disabilities (n=61), only 6 children (10%) did not have problems on either of these 4 aspects of school performance.

Table 3: Impact of NF1 on school performance.

	Patient group % (n)	Dutch population %	Odds Ratio for NF1 (95% confidence interval)
Type of education (n=86)†			
- Special education	37% (32)	9.0%	4.1 (3.1-5.4)**
- School for learning disabled	33% (28)	8.3%	3.9 (2.9-5.3)**
- School for mentally retarded	4.7% (4)	0.6%	7.3 (2.8-19.0)**
- Regular education	63% (54)	91.0%	0.7 (0.6-0.8)**
Repeated grade (n=70)‡	40% (28)		
- in kindergarten	19% (13)	14.3%	1.3
- in primary school	17% (12)	1.9%	9.0**
- in secondary school	4% (3)		
Remedial teaching given (n=75)§			
-Yes	85% (64)		
- In primary school (n=52)	85% (44)	15%	5.6 (5.1-6.3)**
-No	15% (11)	85%	0.2 (0.1-0.2)**
Type of remedial teaching (n=64)			
-Didactical	73% (47)		
-Motorical	42% (27)		
-Logopedical	36% (23)		
-Behavioral	22% (14)		
-Not specified	3% (2)		

** P-value binomial test < 0.0005. †Reference values from Dutch Ministry of Education, Culture and Science (N = 2.597.700)[18]. ‡Reference values from Dutch Inspection of Education[19], Confidence interval not available. §Reference values from Matthijsen et al. (children 8-11 years, N=9.734)[20]. NF1: Neurofibromatosis type 1.

Neuropsychological assessment

Out of the 86 participating children, 62 (70%) consented to a neuropsychological assessment. These children showed a mean Full Scale IQ of 86, which was distributed normally. Mental retardation (Full Scale IQ<70) was present in 11 children (18%). The performance IQ profile showed a dip in the scores for block design and object assembly. Compared with normative

Table 4: Neuropsychological results in learning disability groups.

Neuropsychological Test*	Total NF1 group, n=62; mean (SD)	NoLD group, n=8; mean (SD)	SLD group, n=16; mean (SD)	GLD group, n=17; mean (SD)	ǂ
Intelligence†					
Full Scale IQ	86.2 (15.3)	96.8 (12.1)	97.9 (8.1)	72.6 (7.5)	2,3
Verbal IQ	86.7 (16.2)	98.4 (11.4)	98.2 (10.0)	71.4 (8.5)	2,3
Performance IQ	88.7 (14.7)	95.6 (11.6)	98.4 (12.2)	79.9 (8.7)	2,3
Verbal Comprehension Index	88.5 (14.9)	97.1 (8.8)	99.5 (8.4)	76.0 (9.9)	2,3
Perceptual Organisation Index	88.0 (14.7)	94.6 (10.7)	97.5 (11.2)	79. (10.1)	2,3
Freedom from Distractibility	88.4 (16.8)	102.1 (14.4)	97.6 (14.3)	73.8 (7.2)	2,3
Information	7.8 (2.9)	9.3 (3.4)	9.5 (1.8)	6.1 (2.1)	2,3
Similarities	9.0 (3.2)	10.6 (1.9)	11.4 (2.0)	6.3 (2.2)	2,3
Arithmetic	7.3 (3.9)	10.4 (2.0)	8.3 (4.1)	4.1 (1.8)	2,3
Vocabulary	7.4 (2.7)	9.0 (1.7)	9.2 (1.6)	5.4 (1.8)	2,3
Comprehension	7.9 (2.7)	9.4 (1.8)	9.3 (2.3)	6.3 (1.8)	2,3
Digit Span	8.0 (3.4)	10.1 (3.8)	10.5 (2.8)	5.2 (2.1)	2,3
Picture completion	9.3 (3.5)	9.1 (2.5)	12.3 (3.3)	8.0 (2.9)	1,2
Picture arrangement	9.6 (3.0)	10.5 (2.2)	10.5 (3.0)	8.4 (3.2)	
Block Design	7.1 (2.6)	8.4 (2.2)	8.6 (2.7)	5.8 (2.1)	2,3
Object assembly	7.1 (3.2)	8.1 (3.3)	7.8 (2.5)	6.6 (2.6)	
Coding	9.3 (2.7)	10.5 (2.3)	9.9 (2.6)	8.7 (2.4)	
Mazes	8.2 (3.2)	10.1 (2.6)	8.6 (3.2)	6.7 (2.4)	3
Memory					
Rey AVLT – immediate recall	-0.03 (1.09)	0.04 (1.41)	0.34 (1.01)	-0.53 (1.04)	
Rey AVLT – delayed recall	0.11 (1.04)	0.18 (1.18)	0.37 (0.85)	-0.18 (1.16)	
Rey CFT – delayed recall	-1.64 (0.99)	-1.86 (0.75)	-1.48 (0.83)	-1.54 (0.72)	
Language					
PPVT	0.31 (1.11)	0.93 (0.66)	0.86 (0.65)	-0.26 (1.02)	2,3
Boston Naming test	-1.12 (1.75)	-0.44 (0.98)	-0.24 (1.15)	-1.70 (1.82)	2
Visual-spatial skills					
Judgement of Line Orientation	-1.37 (1.45)	-1.65 (0.94)	-0.56 (1.66)	-1.68 (1.13)	
Rey Complex Figure Test – copy	-1.28 (1.28)	-0.36 (0.82)	-0.79 (0.96)	-1.68 (1.25)	2,3
Beery VMI	-1.16 (0.84)	-0.38 (0.66)	-0.85 (0.66)	-1.53 (0.70)	2,3
Executive skills					
Trailmaking Test A	-0.69 (1.12)	-1.09 (1.24)	-0.69 (0.87)	-0.78 (1.36)	
Trailmaking Test B	-0.58 (1.10)	-1.24 (1.20)	-0.35 (1.19)	-0.86 (1.04)	
Animal naming	-0.08 (1.23)	0.88 (1.48)	0.06 (1.27)	-0.67 (0.91)	3
Wisconsin CST – perseverations	-0.19 (1.13)	-0.06 (0.50)	0.13 (1.28)	-0.84 (1.20)	3
Wisconsin CST – categories	-0.23 (1.18)	0.26 (0.96)	0.02 (1.09)	-0.85 (1.34)	
Attention					
Stroop Color-Word Test – speed	-0.35 (1.94)	-0.60 (1.24)	0.67 (1.74)	-1.15 (1.77)	2
Cancellation Test – speed	-1.02 (1.70)	-1.60 (0.70)	-0.02 (1.60)	-1.67 (1.80)	1,2
Cancellation Test – AF‡	-2.75 (1.49)	-3.34 (0.84)	-2.17 (1.11)	-3.02 (1.92)	1
Cancellation Test – corrections§	1.0 (1.6)	0.9 (1.0)	1.4 (2.4)	0.7 (0.8)	
Cancellation Test – omissions**	15.5 (16.1)	15.3 (6.3)	12.9 (8.6)	20.6 (24.4)	

*Scores are Z-scores (deviation from the mean of a normal population) unless otherwise indicated. †Scores are standard scores (normative Scale and Index scores: mean=100, D=15; subtest scores: mean=10, SD=3). ‡Standard deviation of speed in seconds (no Z-score available; normative score for 12 year-olds: mean=1.7 seconds, interquartile range 2.2-1.5). §Number of corrections (no Z-score available; cutoff for clinical significance: >3 corrections). **number of omissions (no Z-score available; cutoff for clinical significance: >3 corrections).

††Kruskal-Wallis, post hoc Mann-Whitney p<0.05 for: 1 = Comparison No Learning Disabilities – Specific Learning disabilities groups; 2 = Comparison Specific Learning Disabilities – General Learning Disabilities groups; 3 = Comparison No Learning Disabilities – General Learning Disabilities groups.

NF1: Neurofibromatosis type 1, SD: Standard deviation, SLD: Specific Learning Disabilities, GLD: General Learning Disabilities, NoLD: No Learning Disabilities, Rey AVLT: Rey Auditory Verbal Learning Test, Rey CFT: Rey Complex Figure Test, PPVT: Peabody Picture Vocabulary Test III, Beery VMI: Beery developmental test of visual motor integration, CST: Card Sorting Test, AF: attention fluctuations

scores, significant impairments were seen in performance on the visual-spatial skills of line orientation (Judgment of Line Orientation task), visual integration (Rey Complex Figure Test – copy), and visual motor integration (Beery Developmental Test of Visual - Motor Integration), on nonverbal long-term memory (Rey Complex Figure Test – delayed recall), sustained attention (Cancellation Test – speed and attention fluctuations), executive functions (rote memory and divided attention on Trailmaking Test A and B), and expressive language (Boston Naming Test, see table 4, all $p<0.05$). However, children with NF1 scored significantly higher on receptive language (Peabody Picture Vocabulary Test, z-score=0.31, t=2.21, p=0.031). After correction for IQ, the visual spatial-skills and nonverbal long term memory were still significantly impaired. A significant discrepancy between verbal IQ and performance IQ (≥12 points difference) was noted in 35% of the patients, which was not significantly more in favor of verbal (13%) or performance IQ (23%) ($\chi^2=1.64$, p=0.44). Children with a specific comprehensive reading or mathematics disability had a significant higher frequency of intelligence discrepancies (75%, $\chi^2=6.96$, p=0.031, and 70%, $\chi^2=6.92$, p=0.031).

For 41 children, both the neuropsychological test results and the school performance data on all four academic fields were present. Children in the No Learning Disabilities group, although with normal mean IQ (96.8), showed the same dip in their performance IQ profiles (on block design and object assembly) as the entire NF1 group. Compared to normative scores, children in the No Learning Disabilities group also scored significantly lower on nonverbal long-term memory (Rey Complex Figure Test – delayed recall), line orientation (Judgment of Line Orientation task), rote memory and divided attention (Trailmaking Test A and B), sustained attention (Cancellation Test – speed and attention fluctuations; all $p< 0.05$; see table 4).

Disease severity

To test whether there was a relationship between disease severity and cognitive function, we used the Riccardi Scale, modified to exclude cognitive aspects of NF1. A general tendency for lower scores on didactic and neuropsychological tests was observed with increasing severity of

NF1 (Figure 1). In particular, children with minimal NF1 seemed to consistently perform better than children with mild or moderate NF1, whose scores were similar on all tests.

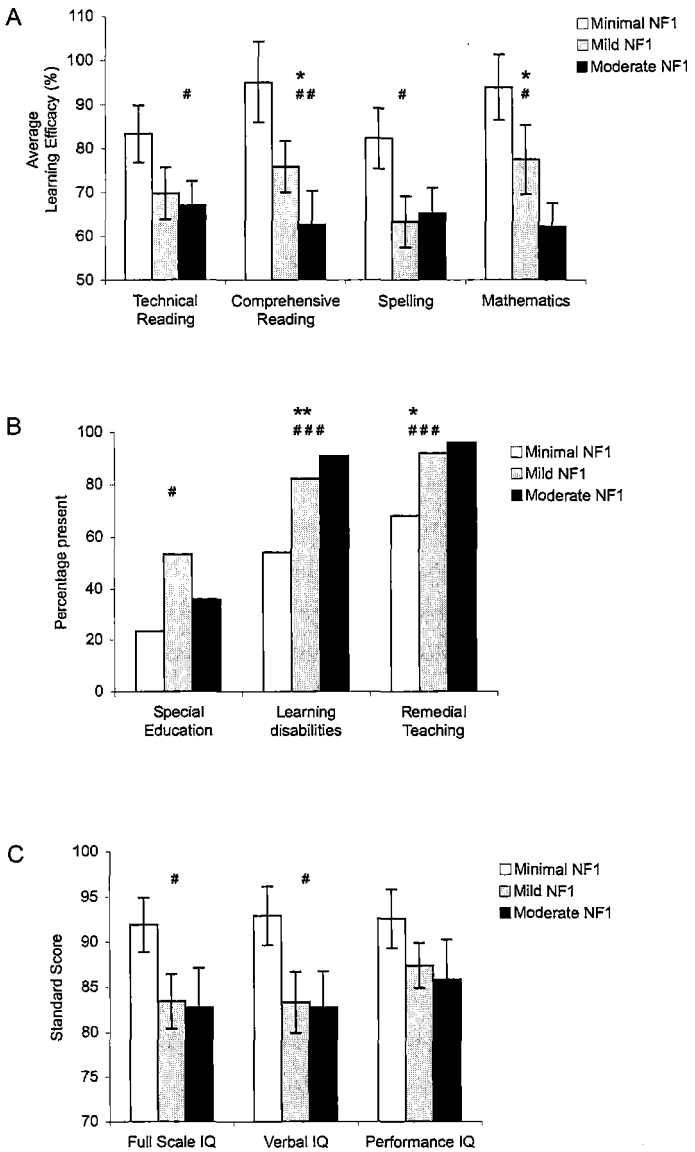

Figure 1: Relationship between clinical severity of Neurofibromatosis type 1 (modified Riccardi Scale) and learning efficacy (A), referral to special education, learning disabilities, and remedial teaching (B), and intelligence (C).
Asterisks indicate statistical significant differences using Analysis of Variance or Kruskal-Wallis between the three groups (* $p<0.05$; ** $p<0.01$). Pound signs indicate statistical significant differences using Student's t-test or Chi-Square test between the minimal NF1 group and mild/moderate NF1 groups (# $p<0.05$; # # $p<0.01$; # # # $p<0.005$). Bars represent standard error of the mean. NF1: Neurofibromatosis type 1.

Indeed, comparison of these two groups revealed that children with mild/moderate NF1 (n=55) have significantly lower learning efficacies than children with minimal NF1 (n=30) for all 4 didactic domains: technical reading (68 versus 83%, t=2.01, p=0.048), comprehensive reading (70 versus 95%, t=2.65, p=0.010), spelling (64 versus 82%, t=2.29, p=0.025) and mathematics (70 versus 94%, t=2.55, p=0.013). In addition, children with mild or moderate NF1 had a significantly higher frequency of learning disabilities than children with minimal NF1 (86 versus 54%, χ^2=8.93, p=0.003), received significantly more remedial teaching (94 versus 68%, χ^2=8.76, p=0.003), and attended special education more frequently (45 versus 23%, χ^2=4.05, p=0.044). The mild or moderate NF1 group had a significantly lower Full Scale IQ (83 versus 92, t=2.19, p=0.032), verbal IQ (83 versus 93, t=2.33, p=0.023), and Freedom from Distractibility Index (85 versus 94, t=2.08, p=0.042), as well as significantly lower scores on IQ subtests arithmetic (p=0.005) and mazes (p=0.028), and test measuring line orientation (p=0.047), divided attention (p=0.006) and receptive language (p=0.048) than the minimal NF1 group. There was no significant difference between familial or sporadic NF1 on any parameter of school performance.

Discussion

To our knowledge, this is the first study in which the impact of NF1 on school performance is determined by combining quantitative data on didactic performance obtained in the school setting with information on special education and remedial teaching. Our results clearly demonstrate that school performance is severely affected in children with NF1. At least 75% of the children with NF1 have one or more learning disabilities in technical reading, comprehensive reading, spelling or mathematics. The learning disabilities are distributed equally over these 4 didactic domains, indicating that NF1 pathology does not cause one specific type of didactic deficit. The high incidence of remedial teaching (85%), special education (37%) and grade repetitions (40%) in our study emphasizes the school problems arising from NF1. Only 10% of the children do not have problems in any aspect of school functioning. School performance and cognitive functioning were found to be substantially more affected in patients with more severe physical features of NF1.

The 75% learning disabilities found in this study group is markedly higher than reported previously (52% learning disabilities)[6], but are in agreement with the 70% total learning disabilities of Brewer et al.[8] The frequency of learning disabilities found in our population reflects actual problems experienced by children with NF1 in their school career. This high

number is likely to be more representative because the didactic tests obtained from the school setting that were used in our study have a higher ecological validity than academic achievement tests used in other studies. The Dutch Student Monitoring System assesses didactic progress of each student 3 times a year throughout the school career and scores are compared to normative grade peer scores. Although a performance of –1 SD as a cutoff for learning disabilities was also used in the studies mentioned above, it is still an arbitrary definition. However, even when using a performance of –2 SD as a cutoff (learning efficacy < 70%), still 63% of the children with NF1 display impairments in one or more didactic domains. Notably, the incidence of 75% learning disabilities could be an underestimation of the real school problems experienced by these children, because we have shown that although some children do not display learning disabilities in their didactic scores, they obtain these scores only in the context of remedial teaching specifically for problems in learning. In addition, children in the No Learning Disabilities group are still significantly impaired in nonverbal long-term memory, executive functions and attention. Mild and specific learning disabilities tend to become apparent when increasing demands on cognitive function can no longer be met. This phenomenon resembles 'growing into deficit'.[21] Thus, children without learning disabilities can grow into specific learning disabilities at an older age. This is supported by the analysis that children without learning disabilities in our study are significantly younger than children with learning disabilities (2.4 years, $p<0.0005$). Our data further indicate that the impact of NF1 on school is not only limited to cognitive function, but encompasses motor function as well. In total, 52% of the children received remedial teaching for physical problems such as motor problems or speech problems.

Disease Severity

The phenotype of NF1 is highly variable, even in patients with identical NF1 mutations, which is proposed to be due to genetic modifiers.[22] The relationship that we found between physical symptoms and cognitive symptoms suggests a common genetic basis for both. So far, this relationship has only been observed for children with seizures,[23] and for patients with a microdeletion.[24] However, the more severe cognitive phenotype of this latter group may be caused by the deletion of genes outside the NF1 gene. The lack of a correlation between disease severity and cognitive function in other studies could be caused by not applying a severity scale,[22] by excluding patients with specific NF1 characteristics (for instance, patients with optic gliomas [25]) or by using global intelligence instead of more detailed cognitive functions.[25]

Neuropsychological testing

We found a markedly higher frequency of mental retardation in our sample than previously reported in other studies on NF1 (4-8%)[7]. However, if we shift the normal distribution curve of IQ to the left to an average IQ of 86 (with a standard deviation of 15) as found in our study, the expected frequency of mental retardation would be 14.3%, which is in accordance with our findings. Interestingly, in our study group only 3 children (4.8%) had both a performance IQ and a verbal IQ of below 70, indicating that not all of the children with a full scale IQ below 70 perform at the level of mental retardation.

Based on the neuropsychological profile of NF1 patients, remedial teaching for learning disabilities in children with NF1 could be tailored to address the specific cognitive functions that are impaired. In addition, weaknesses that are specific to NF1 can be avoided. For instance, many children with NF1 have a poor visual analysis and could benefit from a verbal rather than visual presentation of mathematical problems. The potential value of remedial teaching is illustrated by the observation that most children in the No Learning Disabilities group receive remedial teaching. Thus, despite the observed deficits in the neuropsychological profile of these children, they do not (yet) show deficits in any of the didactic domains. However, as discussed above, there is a fair chance that these deficits will become apparent at older age. Thus, although remedial teaching and special education can ameliorate learning challenges, these interventions are not sufficient to eliminate them.

Our study shows large attention problems in children with NF1. Hyman et al.[6] showed comorbidity of attention-deficit hyperactivity disorder with literacy problems. The importance of attention for technical reading is supported by recent evidence that methylphenidate improves comorbid dyslexia in children with attention-deficit hyperactivity disorder.[26] Methylphenidate has been reported to have favorable effects on attention and behavior in NF1 patients.[27] As noted above, the children in the No Learning Disabilities group show severe attention deficits, which could put them at risk for developing learning disabilities over time. Although these children could potentially profit from timely recognition and treatment with methylphenidate, only one child in this group received medication for attention-deficit hyperactivity disorder, which illustrates the risk to overlook attention problems in children with relatively good school results.

Limitations

The patients participating in this study were selected from the patient group of a university hospital with a specialized NF1 clinic. Potentially, this could result in a referral bias toward children with more severe physical symptoms. However, the large group of children with only minimal NF1 contradicts a referral bias. Also, there was no difference between the group for which a school performance questionnaire was received (n=86) and the group for which it was not (n=28) regarding the distribution of disease severity (p=0.28), age (p=0.86), the frequency of special education (p=0.63) or the frequency of mental retardation (p=0.68). This indicates there was no selection bias in the data from the school performance questionnaire. Finally, the frequency of mental retardation does not differ between the group that consented to neuropsychological assessment (n=62) and the group that did not (n=52) (p=0.14; data from the non-response group was obtained from patient charts).

It should be noted that many school performance questionnaires missed quantitative data on one or more of the four didactic domains. In most of the cases that data were missing, teachers did provide *qualitative* data; however this was not used in our study. Evaluation of these qualitative scores does not suggest a bias toward selectively omitting good or bad school performance data. However, missing data could lead to an underestimation of the amount of learning disabilities, because children who show no learning disabilities but have missing data for some domains (n=7), could still have learning disabilities in the missing domains. Therefore, we have stated that the percentage of learning disabilities we found is *at least* 75%. In the analysis of disease severity, we did not exclude children with a microdeletion because not all children received genetic testing. This could potentially influence the strength of the association found in our study. However, the severity scores of the 4 children in our study that had a known microdeletion were evenly distributed over the severity groups (2 minimal, 1 mild and 1 moderate NF1), making a confounding effect less plausible.

The high number of children with NF1 who receive special education or remedial teaching found in our study population is alarming but is also encouraging because it suggests that the Dutch school system does recognize the need for extra support of children with NF1. Because school systems may be organized differently in other countries, the actual percentage of children receiving special care may vary from country to country. However, the reported odds ratio for receiving special education or remedial teaching should be applicable to all school systems. Therefore, our study can be used as a general guide to counsel parents and teachers to be alert

for problems in learning, motor functioning, speech, and behavior. Awareness of the school problems associated with NF1 may facilitate timely recognition and appropriate support.

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CHAPTER 4

Health Related Quality of Life in children with Neurofibromatosis Type 1: Contribution of demographic factors, disease related factors, and behavior

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Abstract

Objective

We aim to investigate Health Related QOL (HR-QOL) in children with Neurofibromatosis type 1 (NF1) using parental reports and children's self-reports, and to investigate the potential contribution of demographic factors, disease-specific factors, and problems in school performance or behavior.

Study Design

In a prospective observational study, parents of 58 children with NF1 (32 boys, 26 girls, age 12.2 ± 2.5 years) visiting a university clinic, and their 43 children 10 years or older were assessed with the Child Health Questionnaire (CHQ). Potential determinants of domain scores were assessed in three explorative regression models.

Results

Parents reported a significant impact of NF1 on 9/13 CHQ scales, with moderate effect sizes on 8 (General Health Perceptions, Physical Functioning, General Behavior, Mental Health, Self Esteem, Family Activities, Role functioning Emotional/Behavioral, and Parent Emotional Impact). Children report an impact on Bodily Pain, and an above average General Behavior. Multiple CHQ scales were sensitive to demographic factors and behavioral problems, and one to NF1 severity. NF1 visibility and school problems did not influence HR-QOL.

Conclusions

Parents, but not NF1 children themselves, report a profound impact of NF1 on physical, social, behavioral and emotional aspects of HR-QOL. Multiple HR-QOL domains were most sensitive to behavioral problems, which points to an exciting potential opportunity to improve HR-QOL in children with NF1 by addressing these behavioral problems .

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant genetic disease with an incidence of 1 in 3000, half of which are *de novo* cases.[1, 2] The disease is characterized by various progressive neurocutaneous manifestations, including café au lait maculae, axillary freckling, neurofibromas, and Lisch nodules. At pediatric age, possible complications include deformities due to plexiform neurofibromas, neurologic problems, malignancies, endocrine disturbances and orthopedic problems such as scoliosis.[2] However, the most frequent complication of NF1 in children is cognitive impairment, characterized by a low-average IQ, and problems with visual-spatial skills, memory, language, executive functioning and attention.[3, 4] Due to these problems, up to 75% of the NF1 children have learning disabilities and the majority of children needs additional support in the form of special education or remedial teaching.[4] In addition, children with NF1 demonstrate poor social skills, are frequently picked on by peers and have fewer friends.[5-7] Behavioral problems are commonly reported, and include Attention Deficit Hyperactivity Disorder (ADHD) in up to 40%.[5, 6, 8, 9]

As can be expected from the various physical, cognitive and social complications associated with NF1, a below average Quality of Life (QOL) has been reported by NF1 adults.[10, 11] This lower reported Quality of Life was recently confirmed for NF1 children in one study on preschoolers, and one on children aged 7 to 16 including child self-reports.[12, 13] Although NF1 physical disease severity and disease visibility have been related to lower scores on QOL[10, 12, 13], it has not been investigated to what extent the school problems and behavioral problems experienced by children with NF1 contribute to problems reported in QOL.

This study aims to investigate Health Related QOL (HR-QOL) in children and adolescents with NF1 using parental reports and children's self-reports, and to investigate the potential contribution of demographic factors, disease-specific factors, and problems in behavior or school performance.

Methods

Procedure

We performed this prospective observational study in the context of the baseline inventory of a larger study on cognitive functioning among 62 children with NF1 between January 2006 and

March 2007.[14] Participants were recruited from the multidisciplinary pediatric NF1-outpatient clinic of the Erasmus MC – Sophia Children's Hospital Rotterdam, which is a supraregional reference center covering about 3 million citizens. For detailed inclusion and exclusion criteria we refer to our previous publication.[4] For the purpose of the current study, patients were only included if at least the parent HR-QOL questionnaire was completed. Children visited the clinic for neuropsychological testing.[4] During this visit, children and parents received questionnaires on HR-QOL, and an additional questionnaire on behavior, to be completed by the child's teacher. A return envelope was provided to allow for filling out the questionnaire at home. This study was approved by the Erasmus MC – Sophia Children's Hospital Medical Ethical Committee.

Measurements

Health related Quality of Life (HR-QOL)

HR-QOL was assessed with the Child Health Questionnaire (CHQ).[15] This internationally applied generic questionnaire covers physical, psychological and social aspects of quality of life. It comprises a Parent Form (CHQ-PF50; 50 items, 13 domains), to be completed by parents of children from 5 to 18 years old and a Child self-report form (CHQ-CF87; 87 items, 12 domains) to be completed by children from 10 years old themselves. Reference-values and reliability and validity of the CHQ-PF50 and CHQ-CF87 have been determined for the Dutch population.[16, 17] Parallel domains in the CHQ-PF50 and CHQ-CF87 are 8 multi-item scales: General Health Perceptions, Physical Functioning, Bodily Pain, General Behavior, Mental Health, Self Esteem, Family Activities, and Role Functioning – Physical, and 2 single item questions: Family Cohesion and Change in Health. The child form further provides a Role Functioning - Behavior and Role Functioning - Emotion score, and the parent form a Role Functioning - Emotion/Behavior summary score, Parental Time Impact and Parental Emotional Impact scale. Role Functioning refers to limitations in schoolwork or activities as a result of behavioral problems, emotional problems or both. Scale item scores are summed and transformed into scores on a scale of 0 (worst possible health state) to 100 (best possible health state).[15]

Demographic and disease-related factors

All clinical data were registered by an experienced pediatrician of the NF1 team (A. de G-B). Familial or sporadic NF1 was recorded. Socio-economic status (SES) was determined from highest parental occupation or, if not present, highest parental education, and divided into low, middle or high (modified from a standard occupation classification).[18] Based on the last visit to the outpatient clinic, visibility of NF1 when fully dressed was scored according to Ablon,[19] with

mild visibility indicating no visible tumors and unremarkable gait and posture, moderate visibility indicating patients had some visible tumors or skeletal features without noticeable limp, and severe visibility indicating the presence of numerous visible tumors, optic glioma affecting sight, or severe skeletal features with noticeable limp. Physical disease severity was scored according to the most recent version of the Riccardi Scale,[20] modified to exclude cognitive aspects of NF1 in order to be able to assess physical severity and cognitive problems separately. Severity was scored Minimal (when the patient had no features that compromise health, i.e. only harmless cosmetic features such as *café au lait* maculae, freckling and Lisch nodules), Mild (patient had minor medical complications such as ptosis or discrete plexiform neurofibroma), Moderate (patient had complications that are a significant compromise to health, such as paravertebral neurofibromas or low grade glioma) or Severe (medical history of malignancy).

Behavior and School performance

We used the Total Problems score on the standardized Teacher's Report Form (TRF, 118 items)[21] to assess behavioral and emotional problems. The TRF is validated for the Dutch population and provides summary scores for Internalizing (subscales social withdrawal, somatic complaints, and anxiety/depression), Externalizing (subscales rule breaking behavior and aggressive behavior), and Total problems (overall summary score). Items are rated 0 (never true), 1 (sometimes true) or 2 (clearly or often true). Scores are converted to T-scores (mean 50, SD 10), with higher scores indicating more problems.

We used data on school type, learning disabilities and need for remedial teaching, obtained from teacher questionnaires in the ongoing study on cognition,[4] to obtain a 4-level school-scale: 1) No learning disabilities, no remedial teaching, regular education; 2) Learning disabilities and/or remedial teaching, regular education; 3) School for the learning disabled; 4) School for the mentally retarded or severely learning disabled.

Analysis

Data were analyzed in SPSS 12.0. Differences in CHQ scores compared to reference values[16, 22] were assessed using a two-sided independent t-test. For each CHQ domain, effect sizes were calculated compared to reference values[16, 22] in order to evaluate clinical relevance of scores (mean reference – mean NF1)/(square root (pooled SD)). According to Cohen's guidelines, effect sizes from 0.2 to 0.5 were defined as small, from 0.5 to 0.8 as moderate, and >0.8 as large.[23]

Internal reliability of the CHQ domains when applied to the NF1 population was assessed using Cronbach Alpha. To compare children's and parent's ratings, we looked at differences in effect sizes, since absolute differences in scale scores are not only informative of NF1 but also reflect differences observed between parents and children's ratings in the normal population. In addition, to examine the strength of concordance between ratings by parents and children we calculated Intraclass Correlation Coefficients (ICC's), which take into account the individual variability between parent and children pairs. ICC's below 0.4 were considered to reflect poor to fair, between 0.4 and 0.6 moderate, between 0.6 and 0.8 good and above 0.8 excellent agreement.[24] Because the CHQ-CF is constructed for children aged 10 years or older, comparison of children's and parent's ratings is performed on the subgroup of children aged 10 years and older only (n=43).

Using multiple linear regression (enter method) we built three separate models to explore determinants of CHQ domains scores that were significantly affected. Model 1 contained demographic factors (child's age, child's sex, socio economic status of the family, familial/sporadic NF1). Model 2 included disease related factors (NF1 severity and visibility). Model 3 consisted of problems in behavior and school performance (TRF total problems, School Scale). For reliable analysis, scales with less than 5 patients on a subscale (visibility severe, severity severe, School Scale 1) were merged with the closest ranking subscale. The patient with unconfirmed familial NF1 was excluded from analysis of model 2. Reported values are the regression coefficients for the condition, corrected for the other conditions within the model.

Results

The CHQ-PF was received from parents of 58 out of 62 eligible children (response rate 94%). Of these 58 children, 43 were aged 10 years or older. All of these 43 children completed the CHQ-CF (response 100%). In addition, the TRF was received from 54 of the teachers of the 58 participating children (response 93%). The study included four sibling pairs. Patient characteristics are shown in table 1. Complications resulting in a Mild severity score were constipation (n=4), discrete plexiform neurofibroma (n=10), sleep disturbance (n=2), ptosis (n=2), strabismus (n=1), scoliosis (n=1), leg length asymmetry (n=1), deafness (n=1), or a Central Nervous System cyst (n=1). Moderate severity was scored for paraspinal neurofibromas (n=9), diffuse plexiform neurofibroma (n=3), puberty disturbance (n=5), low-grade glioma

(n=3) or pseudo arthrosis (n=1). One child had a Severe severity due to a medical history of Myelo-Dysplastic Syndrome.

Table 1: Characteristics of children with NF1 (N=58) and their parents.

Characteristic	N (SD)	%
Demographic factors		
Age child (mean)	12.2 (2.5)	
Male sex	32	55
Socio-Economical Status		
Low	21	36
Middle	17	29
High	20	35
Mode of inheritance		
Familial	22	38
Sporadic	35	60
unconfirmed	1	2
Comorbid conditions	7	12
Disease-specific factors		
Ablon Visibility Scale		
Mild	44	76
Moderate	10	17
Severe	4	7
Modified Riccardi Scale		
Minimal	13	22
Mild	23	40
Moderate	21	36
Severe	1	2
School performance and behavior		
School Score†		
1 – No LD, no RT	3	6
2 – LD and/or RT, regular education	27	49
3 – School for the learning disabled	18	33
4 – School for the mentally retarded	7	13
Behavior (TRF Total Problems, T-score)	56.2 (8.6)*	

*p<0.05 compared to normative scores (mean = 50, SD = 10).

†for 3 patients on regular education, information on learning disabilities or remedial teaching was missing.

NF1, Neurofibromatosis type 1; LD, Learning Disabilities; RT, Remedial Teaching; TRF, Teacher's Report Form.

Reported CHQ domain scores are shown in table 2. Parents rate their children's HR-QOL as significantly lower than reference values for 9 out of 13 domains, of which 8 with a moderate effect size (General Health perceptions, Physical Functioning, General Behavior, Mental Health, Self Esteem, Family Activities, Role Functioning Emotional/Behavioral, and Parent Emotional

Table 2. Parent- and child reported CHQ scores for children with NF1.

	Parental Report (N=58)					Child Report (N=43)				
	NF1		Reference†			NF1		Reference‡		
Scale (range 0-100)	Mean	(SD)	Mean	(SD)	Effect size	Mean	(SD)	Mean	(SD)	Effect size
General Health Perceptions	71.9**	(17.5)	82.9	(13.4)	-0.7	72.7	(16.2)	74.6	(15.9)	-0.1
Physical Functioning	94.5**	(9.5)	99.1	(4.3)	-0.6	95.7	(8.9)	96.8	(5.4)	-0.1
Bodily Pain§	81.2	(17.4)	85.7	(17.2)	-0.3	71.4*	(27.5)	78.2	(19.5)	-0.3
General Behavior	69.5**	(17.6)	78.5	(13.1)	-0.6	86.8*	(8.8)	83.6	(10.2)	0.3
Mental Health	75.3**	(14.7)	81.4	(12.1)	-0.5	79.8	(12.8)	78.2	(13.0)	0.1
Self Esteem	72.9**	(12.8)	79.2	(11.0)	-0.5	76.9	(13.5)	75.4	(12.5)	0.1
Family Activities	77.8**	(23.1)	91.5	(11.9)	-0.7	83.0	(17.5)	nr	nr	nr
Family Cohesion	67.2	(23.8)	72.2	(19.4)	-0.2	75.7	(26.7)	75.7	(23.1)	0.0
Change in Health§	59.5	(16.8)	nr	nr	nr	66.1	(22.6)	nr	nr	nr
Role Functioning – Physical	94.0	(15.2)	95.8	(15.6)	-0.1	96.1	(11.3)	96.5	(11.6)	0.0
Role Functioning – Emotional	na	na	na	na	na	92.0	(14.4)	92.3	(16.8)	0.0
Role Functioning – Behavioral	na	na	na	na	na	93.0	(17.1)	91.4	(13.7)	0.1
Role Functioning – Emotional / Behavioral	90.4**	(17.0)	97.9	(7.2)	-0.6	na	na	na	na	na
Parental Emotional Impact	73.0**	(21.1)	86.3	(15.2)	-0.7	na	na	na	na	na
Parental Time Impact	87.5**	(18.2)	94.0	(13.0)	-0.4	na	na	na	na	na

*=$p<0.05$, ** $p<0.01$ for comparison NF1 scale scores to reference scale scores.

†Reference population: parents of Dutch schoolchildren 5-13 years, N=353.[15]

‡Reference population: Dutch schoolchildren 9-17 years, n=444.[16]

§=Child report n=42.

nr: No reference values available, na: Not applicable.

Impact). Parental reports for children older than 10 years were similar to parental reports for younger children. Children rated their HR-QOL not different from reference values, except for significant lower scores on Bodily Pain and significant higher scores on General Behavior, both with a small effect size. For this NF1 population, internal reliability of the CHQ parent and children scales was very good (Cronbach's Alpha 0.73-0.93) except for General Health Perceptions, Physical Functioning and Self Esteem reported by parents (0.54 to 0.66).

As shown in figure 1, we observed moderate to large differences between parental and children's ratings on the majority of the domains (differences more than 0.5 effect size in 5 out of 8 scales depicted), with generally higher scores, indicating less problems, reported by children than by their parents. ICC's between parent and children's ratings ranged from poor (2 scales) to moderate (4 scales) and good (2 scales). The size of the gap between effect sizes reported by parents and children did not always match the level of concordance. On Physical Functioning, the difference between parents and children was moderate (0.5 effect size), but there was a good concordance (0.72). This should be interpreted as follows: both parental and child reported CHQ scores deviate from reference values in the same direction (i.e. both lower scores) but nevertheless there is a moderate absolute difference in the domain scores reported by parental and children. For General Behavior, the difference between parental and children reports is large (0.9 effect size) and the concordance is poor (0.30), indicating a high variability between parental and children's reports. Family activities (ICC 0.59) and Change in Health (ICC 0.36) are not depicted in figure 1 because no reference values were available.

To explore which determinants are related to HR-QOL scores, we performed a regression analysis in three models: demographic factors, disease-related factors, and problems in school performance or behavior (table 3). Exploration of demographic factors in model 1 revealed that high SES contributes negatively to the score for Bodily Pain in children. For parents, we found a significant positive impact of male sex on Parent Time Impact, and a positive influence of familial NF1 (on Self Esteem). Age of the child did not influence CHQ domain scores. In model 2 (disease-related factors), we observed a negative relationship between severity and General Health Perceptions (significant for moderate versus minimal severity), but no significant influence of NF1 visibility. In model 3, multiple CHQ scales were sensitive to behavioral problems reported by teachers. Total problems on the TRF had a negative impact on General Health, General Behavior and Parent Time Impact. School performance did not influence any CHQ domain score.

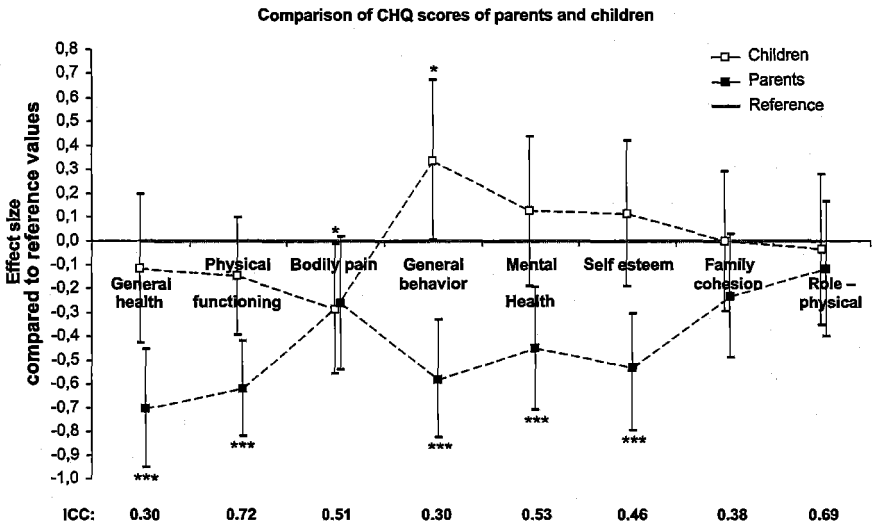

Figure 1: Comparison of CHQ scores of NF1 parents and children.

Error bars represent 95 percent confidence intervals. Differences compared to table 2 are due to rounding.

CHQ: Child Health Questionnaire, NF1: Neurofibromatosis type I, ICC: Intraclass Correlation.

Discussion

We studied the impact of NF1 on HR-QOL in a large group of children with NF1, using parental ratings complemented with children's self reports. Parents report that children with NF1 experience substantial problems compared to healthy children on 9 out of 13 CHQ domains, with moderate effect sizes on 8 domains. We observed profound impact of NF1 on parents themselves and on their family life. In contrast, we observed that children with NF1 report lower scores on Bodily Pain only, and report above average scores on General Behavior. We measured substantial differences between the effect sizes of parental and children's ratings on the majority of cross-compared scales. Although parents usually reported larger impairments than children, their scores tend to correlate. Social-economic status, sex, familial NF1, NF1 severity and in particular the presence of behavioral problems influenced several CHQ domains. None of the evaluated determinants influenced child reports for General Behavior, or parental reports for Physical Functioning, Role Functioning Emotional/Behavioral, Mental Health or Family activities.

The substantial impairments over multiple HR-QOL domains reported by parents of NF1 children in our study confirm results of other studies using parental reports of QOL in children with NF1[12, 13], and self reports of NF1 adults.[10, 13] In our study, children reported lower scores

Table 3: summary of outcome explorative regression analysis on Child Health Questionnaire (CHQ) scales

	CHILD SELF REPORT		PARENTAL REPORT								
	BP	GB	GH	PF	GB	PT	PE	REB	MH	SE	FA
MODEL 1											
Age child											
Gender						10.8±4.7*					
SES (high)	-26.6±10.2*										
Familial NF1						8.9±5.1§	11.2±6.0§			7.3±3.6*	
MODEL 2											
Severity (mod./severe)			-14.1±6.4*								
Visibility											
MODEL 3											
Behavior (TRF)†			-0.6±0.3*		-0.7±0.3*	-0.7±0.3*					
School Scale											

Values (regression coefficients ± SD) represent difference in CHQ scale score in points (1-100), per unit of increase in score for the independent variable (continuous variables), or compared to the reference category of the variable (female sex, low SES, minimal severity, school scale category I/II, spontaneous NF1, mild visibility).

+: positive impact, –: negative impact on CHQ score, empty box: no association.

*$p<0.05$, §$p<0.1$.

†Higher scores on the TRF indicate more behavioral problems.

Mod.: moderate, BP: Bodily Pain, GB: General behavior, GH: General health perceptions, PF: Physical functioning, PT: Parental time impact, PE: Parental emotional impact, REB: Role functioning – emotional / behavioral, MH: Mental Health, SE: Self esteem, FA: Family activities, SES: Socio-economical Status, NF1: Neurofibromatosis type 1, TRF: Teacher's Report Form.

on one HR-QOL domain only, which is in contrast to the only other study that has investigated children's self ratings of QOL[13] , showing problems in motor, cognitive, social and emotional domains of the TNO-AZL Child Quality of Life Questionnaire (TACQOL). This discrepancy with our results may be explained by methodological differences between the CHQ and TACQOL. CHQ scores are based on the reported frequency of problems [15] , whereas TACQOL domain scores are based on the emotional distress due to the problem rather than the frequency.[25] Possibly, children with NF1 less frequently report or perceive encountered problems than parents, but do report emotional distress over these problems. In our study, we preferred the use of the CHQ because it incorporates 4 domains to assess the impact of a disease on the parents themselves and on the family as a whole, which is not covered by the TACQOL.

The striking above average self-ratings of General Behavior by children are refuted by the substantial impairments in behavior reported by teachers on the TRF, but also by objective measurements of attention reported for this patient group in our previous study.[4] This over-estimation is in line with reports of above average self-concept in NF1 adults,[26] above average self-perceived academic achievement[27] and social skills[7] in NF1 children, and discrepancies between child and parent perceived NF1 disease severity.[28] Together, these reports strongly suggest that children with NF1 have problems in forming or reporting an accurate self-concept. Over-estimation in deficient area's could be related to factors observed more generally in children with learning disabilities or ADHD, such as self-protective mechanisms,[27, 29, 30] or to poor cognitive skills in general.[31] Alternatively, NF1-specific cognitive impairments might also include impairments in self percept *an sich.* In addition to over-estimation of General Behavior, the paucity of problems reported by NF1 children sharply contrasts to the substantial problems reported by parents on multiple CHQ domains. Large differences between parental and child ratings, with higher ratings in children, have been widely reported in other chronic conditions, such as ADHD and cerebral palsy.[32, 33] Interestingly, in ADHD children, discrepancies between parental and child self reports increased with ADHD symptoms of inattentive and combined ADHD.[32] Since the vast majority of ADHD in NF1 is of these subtypes[8], this may partly explain differences between parental and children's ratings in our study.

We observed sensitivity of CHQ domain scores to several of the potential determinants. The negative impact of SES on CHQ domain scores may be explained by higher expectations for their child of parents with higher SES, and the experienced discrepancy between parental expectations and the actual level of functioning of their child. Contradictory results however,

were observed in younger NF1 children, where educational level (not occupation) of the respondent contributed to higher HR-QOL on 5 out of 11 domains, as well as in general population studies.[12, 34] . The positive impact of familial NF1 on CHQ scores is also observed in the study on toddlers.[12] Experience with NF1 in the family may lead to better coping styles, or less recognition of problems in these domains. We could not reproduce the reports of previous studies on NF1 children of an impact of severity and visibility on the emotional domains,[13] of parent-rated severity on Parent Emotional Impact, [12] and visibility on General Health Peceptions.[12] However, in the latter study,[12] both disease severity and HR-QOL were scored by the parents themselves. Parental perspectives influencing both severity score and HR-QOL score could seriously confound the measured relationship. Substantial impact of both severity and visibility on QOL is reported by NF1 adults using self-scored[11] and physician scored[10] severity and visibility.[10, 11] NF1 is a progressive disease, and many children do not yet display the cutaneous signs adults do. Therefore, it may be more informative to assess the impact of visibility and severity of NF1 on QOL in adults.

Our study is the first to address behavioral problems and school problems as potential determinants of HR-QOL. Our explorative regression analysis shows that problems in the behavioral domain are important determinants of HR-QOL scores. The negative impact of behavioral problems seems plausible considering the demand put on parents of children with behavioral disturbances. The sensitivity of multiple HR-QOL domains to behavioral problems underlines the importance of adequately managing the frequent behavioral problems of children with NF1, for instance with stimulant medication,[35] social training programs, and better education of families. So far, studies have only focused on fixed factors influencing HR-QOL such as familial NF1, sex, SES and disease severity. Thus, our study offers a first potential handhold for improving HR-QOL. The effect of behavioral therapies on HR-QOL in NF1 should be addressed in future studies.

The lack of an impact of school performance on any CHQ domain may indicate that parents and children do not perceive school problems to be of influence on their quality of life. Considering the problems with self-perception that have been proposed in NF1, this observation in children self-reports is not surprising. However, why parents do not seem to incorporate educational performance into HR-QOL ratings is not clear, in particular under the consideration that learning disabilities are the most common complication of NF1 at pediatric age.[3] Possibly, this observation reflects a good acceptance of or resignation in school problems in parents of children with NF1. School problems, however, may influence QOL later on in life,

as they negatively influence educational level, job opportunities and SES. This has not been investigated yet.

There are several potential limitations to our study. First, it should be noted that children participated in the current study in the context of the baseline inventory of a larger study on cognition and NF1. [14] This may have resulted in a highly motivated group of families. The children that participated in the current study (n=58) did not differ significantly from the total group eligible for the larger study (n=114) on age, sex, frequency of mental retardation, or disease severity (all $p \geq 0.3$), indicating that they are representative for the total eligible group. Second, the lack of Dutch reference values for the CHQ-PF of children older than 13 years may limit the interpretation of our data. However, parental reports for children up to 13 years did not significantly differ from reports of parents of children older than 13 years. Finally, our study was not sufficiently empowered for subgroup-analysis on determinants of parent-child discrepancies, or maternal or paternal effects on CHQ scores.

Conclusion

Parents report a profound impact of NF1 on physical, social, behavioral and emotional aspects of HR-QOL. These findings underline the importance of multidisciplinary care of children with NF1, encompassing not only physical but also social-emotional and behavioral assessment and support. The substantial negative impact of behavioural problems on HR-QOL domains points to an exciting potential opportunity to improve HR-QOL in children with NF1 by addressing these behavioral problems. The fact that the children in our study report only minimal impact on HR-QOL supports a deficit in self-perception in children with NF1, and emphasizes the importance of cross-informant comparison of HR-QOL reports in order to obtain a comprehensive overview of the impact of a disease on HR-QOL.[36]

List of abbreviations:

NF1, Neurofibromatosis type 1; CHQ, Child Health Questionnaire (PF, Parent Form; CF, Child Form); HR-QOL, Health Related Quality of Life; QOL, Quality of Life; BP, Bodily Pain; GB, General Behavior; GH, General Health Perceptions; PF, Physical Functioning; PT, Parental Time Impact; PE, Parental Emotional Impact; REB, Role Functioning – Emotional /Behavioral; MH, Mental Health; SE, Self Esteem; FA, Family Activities; SES, Socio-economical Status; TRF, Teacher's Report Form; ICC, Intraclass Correlation Coefficient; TACQOL, TNO-AZL Child Quality of Life Questionnaire; ADHD, Attention Deficit Hyperactivity Disorder.

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The eyelink camera (left) and the prism adaptation setup (right).
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CHAPTER 5

Motor learning in children with Neurofibromatosis type 1

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Abstract

Purpose

Neurofibromatosis type 1 (NF1) is characterized by various neurocutaneous symptoms and cognitive impairments. In addition, children with NF1 have frequently been reported to display problems in fine and gross motor performance. However, their alleged deficits in motor learning capacities have so far not been investigated systematically.

Methods

We investigated motor performance and motor learning in 70 children with NF1 and 19 healthy age-matched controls (8-16 years) using various quantitative tests. We used the Beery Developmental test for Visual-Motor Integration (Beery VMI) to assess fine motor performance and visuo-motor integration controlled by mainly cerebral processing, and paradigms for saccadic eye movement adaptation and prism-induced hand movement adaptation to assess motor performance and motor learning capacities controlled by mainly cerebellar processing.

Results

NF1 children scored significantly lower on the Beery VMI, showing problems in both visuo-motor integration as well as in fine motor coordination. While no significant impairments were observed in motor performance of either eye or arm movements, NF1 children did show deficits in motor learning during prism-induced hand movement adaptation. In contrast, saccadic eye movement adaptation appeared not to be affected in NF1. No correlation was observed between scores on any of the three paradigms assessed.

Conclusions

Taken together, our results suggest that the motor problems of children with NF1 in daily life may partly be related to deficits in motor learning. These behavioral deficits may be caused by aberrations within specific regions of the cerebellum and cerebrum, but not by a ubiquitous malfunctioning of these brain regions as a whole.

Introduction

Neurofibromatosis type 1 (NF1, incidence 1:3000)[1] is an autosomal dominant disease caused by a mutation in the gene for neurofibromin on chromosome 17q11.2. NF1 is characterized by a variety of neurocutaneous symptoms and cognitive problems, the latter resulting in a lowered mean IQ and a variety of school problems.[2] In addition, many NF1 patients display impairments in fine and/or gross motor function, and over 40 percent of the NF1 children receive remedial teaching to alleviate or improve motor performance.[2] Fine motor problems are reported in areas of fine motor coordination, fine motor speed, and steadiness.[3, 4] One of the neuropsychological tests consistently reported to be impaired in NF1 patients is the Beery Developmental test for visual-motor integration (Beery VMI[5]), a test for fine motor coordination and the integration between the visual-perceptual and motor abilities.[2, 6-8] Gross motor problems observed in NF1 include hypotonia and problems with motor coordination, balance and gait.[3, 7, 9]

Although it is likely that the fine and gross motor problems in NF1 arise from deficits in a network of brain areas, the cerebellum could be of particular interest in NF1. The involvement of this particular brain structure in NF1 is suggested by behavioral, radiological, and molecular studies of NF1. First, although NF1 patients are not clearly ataxic, the frequently reported clumsiness in movements [10, 11], could be related to deficits in the vermis, intermediate or lateral zones of the cerebellum.[12] Second, the cerebellum is one of the predominant sites for NF1 related hyperintensities visible on T2-weighted MR images, which have been related to impairment of fine motor skills.[3] Third, NF1 specifically seems to affect GABAergic neurons,[13-15] and the cerebellar GABA-agric Purkinje neurons are among the highest neurofibromin expressing neurons in the brain.[16, 17]

The cerebellum plays an important role in motor performance, but also in motor learning, which refers to the ability to continuously adapt movements to optimize performance, a task which requires neuronal plasticity.[18-24] The motor learning capacities of children with NF1 have not been investigated so far. In the present study we quantitatively assessed motor performance and motor learning in a large group of children with NF1.

We assessed fine motor performance using the Beery VMI test, and cerebellar-mediated motor performance and motor learning using tests on eye movement and hand movement control, which are affected in patients with cerebellar deficits.[23, 25-30] Performance and plasticity of saccadic eye movements was examined in a saccade adaptation paradigm,[28] which assesses the

gradual modification of the amplitude of saccadic eye movements induced by a systematic change in the visual environment.[19] Performance and plasticity of hand movement control was assessed using prism adaptation, which refers to the modification of hand movement trajectories in response to visual displacement of the environment induced by wearing prism goggles.[31] We hypothesized that motor learning capacities in children with NF1 are affected.

Methods

Subjects

70 children with NF1 (age 12.3 ± 2.5 years, 36 boys, 34 girls) and 19 healthy control children (age 10.7 ± 2.1 years, 6 boys, 13 girls) participated in this study. Children with NF1 were recruited from the patient group attending the NF1 outpatient clinic of the Erasmus MC – Sophia Children's Hospital in Rotterdam. Some of these children participated in this study in the context of a larger study of NF1 and cognition.[2] Inclusion criteria were NF1 diagnosis according to the criteria of the National Institutes of Health[32] and informed consent from the parents and from the children aged 12 years and older. Exclusion criteria were segmental NF1, pathology of the CNS (other than asymptomatic gliomas), deafness, severely impaired vision, use of anti-epileptics, inefficient production or comprehension of the Dutch language, and severe mental retardation (IQ below 48). The control subjects were children of employees of the Erasmus MC – Sophia Children's Hospital. The study was approved by the Medical Ethical Committee of the Erasmus MC.

Procedure

Subjects participated in three tasks the Beery VMI, a Saccade Adaptation test and a Prism Adaptation test.

Beery VMI - Visual Motor Integration

Fine motor coordination and visual motor integration was assessed with the Beery VMI task,[5] in which children have to imitate or copy up to 30 geometric forms with increasing complexity using paper and pencil. The test was stopped when a child makes more than two errors in a row. Copying errors were marked if they reflected problems in fine motor coordination, rather than a pure visual-spatial problems. The task is specifically designed for children and takes about 10 minutes. Beery VMI scores were standardized for age and sex using normative data for the general population.[5] Differences between the two groups were assessed using Kolmogorov-Smirnov tests.

Saccade Adaptation – Eye movement control

Performance and plasticity of saccadic eye movements was assessed in a classical backward saccade adaptation paradigm.[30, 33] Subjects were seated 70 cm in front of a 21-inch computer screen. This experiment took place in complete darkness. A red filter covered the computer screen to eliminate all light emitted by the monitor other than the visual stimuli. Binocular eye position was recorded using infrared video-oculography (EyeLink 2.04, SensoMotoric Instruments, Berlin, Germany) at a sample rate of 250 Hz.[30, 34] Eye position was calibrated with the built-in 9 points calibration routine. A chin rest ensured a stable position of the head and head movements were monitored using the built-in head-tracking camera.

The saccade adaptation paradigm consisted of three distinct phases: 20 baseline trials, followed by 100 adaptation trials, and 20 extinction trials. In all phases the subjects were instructed to look at a single red dot (0.5 degrees of visual angle in diameter) that jumped from left to right. Each trial started with the dot being displayed at 7.5 degrees of visual angle on the left side from the center of the screen. After fixation the dot was removed on the left and subsequently displayed 7.5 degrees from the center on the right side of the screen, evoking a primary saccadic eye movement from left to right with a target amplitude of 15 degrees. In the baseline and the extinction trials the dot remained on the right side of the screen for 1.5 seconds after which the next trial was started. In the adaptation trials the dot on the right stepped 3 degrees to the left, i.e., 20 percent of the initial target amplitude backwards, during the saccadic eye movement toward it.

The amplitude of the primary saccade was determined for each of the 140 trials. Trials were discarded when the primary saccade did not start on the left side, was not directed toward the target on the right, or had an amplitude of less than 8 degrees. For all trials, the saccadic Gain was defined as the amplitude of the primary saccade divided by the target amplitude (15 degrees), so that a gain of 1 reflects a saccade that lands directly on target.

For each subject, the Baseline Gain was calculated as the average of the gains of the primary saccades made in the 20 baseline trials, and the Adapted Gain as the average of the gains of the last 20 trials in the adaptation phase. For each subject, the saccadic Gain Change was calculated as the difference between Adapted Gain and Baseline Gain. Saccadic Variability in the baseline and adapted phase was defined as the within-subject standard deviation of the primary saccadic gains in these phases.

We defined saccade adaptation significant in an individual, when the Gain Change was larger than the average minus 1 standard deviation of the control group combined with a significant ($p<0.01$) difference between Baseline and Adapted Gains. Participants were excluded from further analyses if the average baseline gain or the baseline saccadic variability was an outlier or extreme value (value more than 1.5 interquartile ranges below the 25th or above the 75th percentile).

Prism Adaptation – Hand movement control

The performance and plasticity of hand movement coordination was determined in a prism adaptation experiment.[28] Subjects were seated in front of a digitizing tablet (Ultrapad A2, WACOM Technologies Corporation, Vancouver, WA, USA). The target (a small cartoon picture) was projected from above on a see-through mirror, so that it seemed to be positioned on the tablet 20 cm straight ahead of the subject, while the hand was also visible. Visual feedback of hand position could be blocked by putting an opaque plate below the mirror, so that the target was still visible through the mirror but the hand below the mirror could no longer be seen (see van der Geest et al.[28] for details of the setup).

The experiment consisted of four phases. In all phases subjects had to move the pen a number of times from a starting position at the left bottom of the tablet (17 cm from the center) towards the position of target over a movement distance of 26 cm with an angle of 50 degrees. In the practice phase (phase 1) the subject had to move the pen towards the target 10 times while they could see their hand (visual feedback). In the pre-adaptation phase (2) the subject had to move the pen 10 times without visual feedback. In the adaptation phase (3) the subject wore prism glasses that shifted the visual world 10 degrees to the right. Subjects had to move the pen 30 times to the target and two additional practice-targets positioned about 17cm to the left and right of the original target. In this phase they could see their hand again, so that the position of the hand and target could be visually aligned. Before the post-adaptation phase (4), the glasses were removed and subjects had to move the pen 10 times without visual feedback.

The end-position of each hand movement across the tablet toward the target was marked manually. The movement angle (in degrees) and the movement distance (in cm) was calculated from the straight line between start- and end-position of the movement. For each subject, the averages and standard deviations of the movement angles and distances in the baseline phase, the pre-adaptation and the post-adaptation phase were determined. To assess the effect of

wearing prism glasses (prism adaptation, also called the after-effect[24]), the change in average movement angle (Angle Change) between the pre- and post-adaptation phase was calculated. We defined prism adaptation significant in an individual, if the change in movement angle was larger than mean minus 1 standard deviation of the controls and the difference between the average pre- and post adaptation angle was significant ($p<0.01$). Subjects had to hold the pen in their dominant right hand. Therefore, seven NF1 children and one control child who were left-handed were not eligible for this task.

To assess motor performance we compared the Beery VMI scores, baseline Saccadic Variability and variability in hand movement angle in the pre-adaptation phase between the two groups. To assess motor learning we compared changes in saccadic gain and changes in movement angles between the two groups. Statistical differences were assessed non-parametrically using Mann Whitney, Chi-Square, and Kolmogorov-Smirnov tests. Spearman correlations between Beery VMI scores and the motor performance and motor learning measures, and age were calculated.

Results

Beery VMI

Beery VMI scores (Figure 1A) were significantly lower in the NF1 group (84 ± 13, $n=70$) than in the control group (102 ± 14, $n=19$, absolute extreme difference = .67, $Z=2.58$, $p<0.001$). Control children completed more items than NF1 children (on average 22.3 ± 2.0 versus 19.6 ± 3.9, absolute extreme difference .371, $Z = 1.44$, $p < 0.05$) before the test was stopped. In the copying errors made in the NF1 group, but also in the control group, visual-spatial problems as well as problems in fine motor coordination were observed (see figure 1B). In the NF1 group about 50% of the copying errors were related to problems of fine motor coordination, rather than to pure visual-spatial problems, which was, however, not significantly different from controls.

Saccade adaptation

70 children with NF1 and 19 control children performed the saccade adaptation test. 17 NF1 children and seven controls were excluded from analysis because of technical failures, including eye tracking difficulties, making too large head movements and making too few saccades for proper analysis.

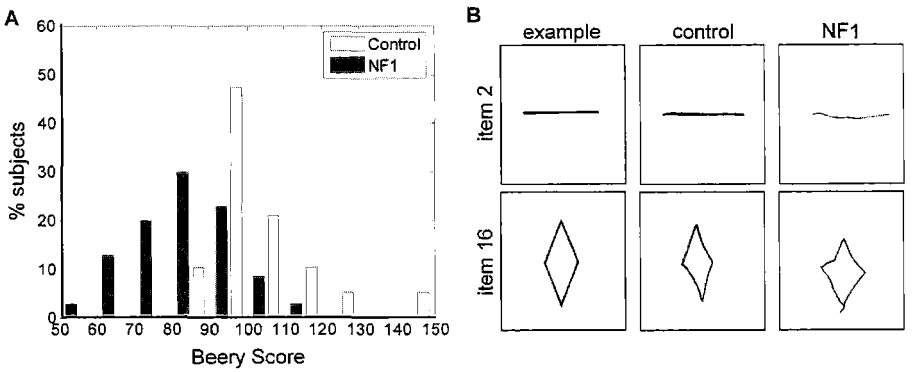

Figure 1: Beery VMI.

Panel A shows the distribution of Beery VMI scores in 70 NF1 children and 19 age-matched controls; panel B shows examples of Beery VMI performance of 2 NF1 children and of 2 age-matched control children with around average Beery VMI score for their respective groups. Items illustrating pure motor problems in these 2 NF children were selected. Performance on item 2 is shown for a male NF1 child (age 14.6y, score 79) and a male control (age 14.3y, score 97). The NF1 child drew an unsteady line, had a weak pencil stroke, and there was an indication of a very discrete tremor. Item 16 is shown for a male NF1 child (age 10.9y, score 84) and a female control (age 10.5y, score 103). The NF1 child shows a general delay in fine motor development and performs around developmental age 5.4y on this item.[5] Note the slip of the pencil at the end of the movement.

We observed no differences in baseline saccadic performance between the remaining 53 children with NF1 (28 boys, 25 girls, 12.6 ± 2.3 years) and controls (2 boys, 9 girls, 10.8 ± 2.1 years). Specifically, the number of correct primary saccades in the 140 trials (122 ± 9 for NF1 vs. 124 ± 9 for controls, p=0.5), the baseline Saccadic Gains (0.91 ± 0.08 versus 0.93 ± 0.04, p=0.4) and baseline Saccadic Variability (0.10 ± 0.04 versus 0.08 ± 0.02, p=0.2) did not differ between the two groups (figure 2A).

Saccadic adaptation was also not different in NF1 children compared to controls (figure 2B) with respect to the size of the Adapted Gains (0.78 ± 0.10 for NF1 vs. 0.78 ± 0.10 for controls, p=0.9) and the adapted Saccade Variability (0.09 ± 0.03 versus 0.09 ± 0.02, p=1.0). The saccadic Gain Change between baseline and the end of the adaptation phase was also not significantly different between NF1 children and controls (0.12 ± 0.08 versus 0.15 ± 0.09, p=0.3). The proportion of subjects with a significant Gain Change (Gain Change >0.06 as derived from the control group) was the same in the two groups (29 out of 53 NF1 children (55%) versus 7 out of 11 controls (64%), χ^2=0.294, p=0.6). The distribution of individual Gain Changes was also not significantly different (absolute extreme difference = 0.235, Z=0.709, p=0.7, figure 2C).

The gain changes were not related to age in NF1 children or controls (R=0.04, p=0.8 for NF1 and R= -0.13, p=0.7 for controls).

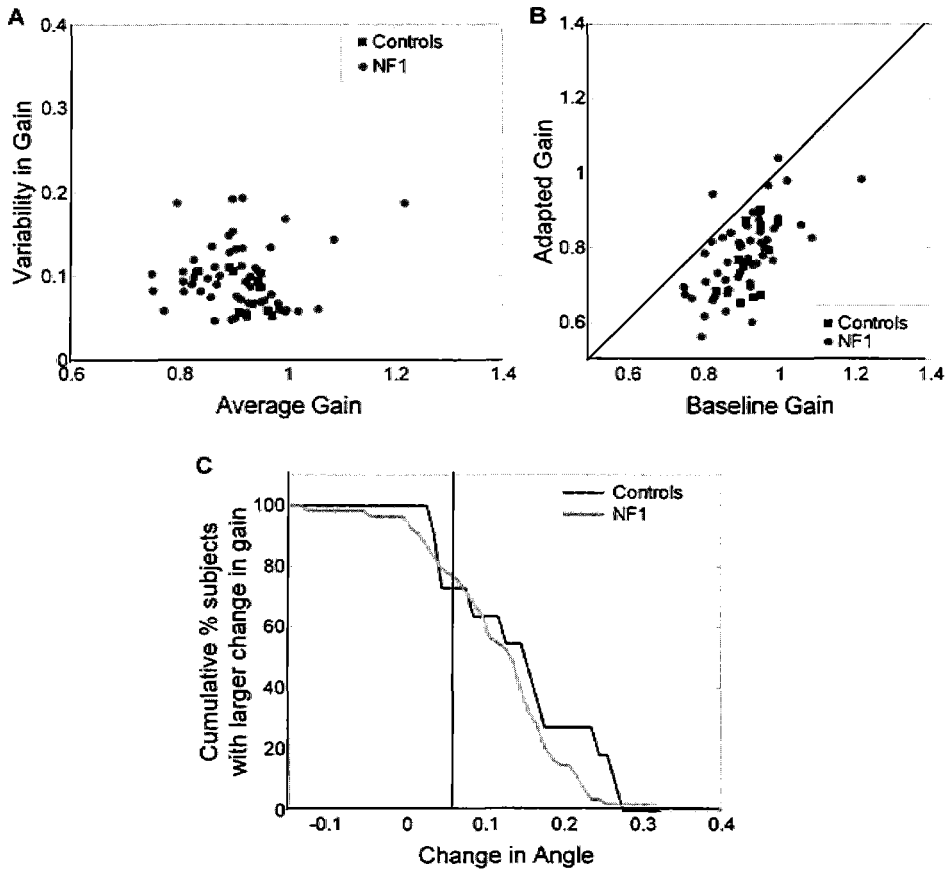

Figure 2: Saccade adaptation.

Panel A shows the variability versus the average of the baseline saccadic gains of 53 NF1 children and 11 age-matched controls; each dot represents one individual subject. Panel B shows the adapted gain versus the baseline gain for these children; the oblique line is the unity line. Panel C shows the cumulative distribution of the Gain Changes in the NF1 and control groups. The vertical line (at Gain Change = 0.06) indicates the cut-off for point significant saccade adaptation.

Prism adaptation

63 right-handed NF1 children and 18 right-handed control children were eligible for the prism adaptation task. Seven NF1 children were excluded from analysis because of technical problems including not understanding or adhering to task instructions. All remaining 56 children with NF1 (29 boys, 27 girls, 12.3 ± 2.4y) and 18 controls (5 boys, 13 girls, 10.6 ± 2.2y) were able to make accurate goal-directed hand movements towards the target. As expected, for both groups

the movement angle was about 50 degrees and the movement distance was about 26 cm when children could align their hand visually with the target in the baseline phase. Without visual feedback (pre-adaptation phase) both groups became less accurate but no difference between the two groups was observed (movement angle: 56.8 ± 3.2 degrees in NF1 vs. 55.6 ± 2.8 degrees in controls, p=0.2; distance: 24.0 ± 2.3 cm in NF1 vs. 24.0 ± 2.0 cm in controls, p=0.9, see figure 3A).

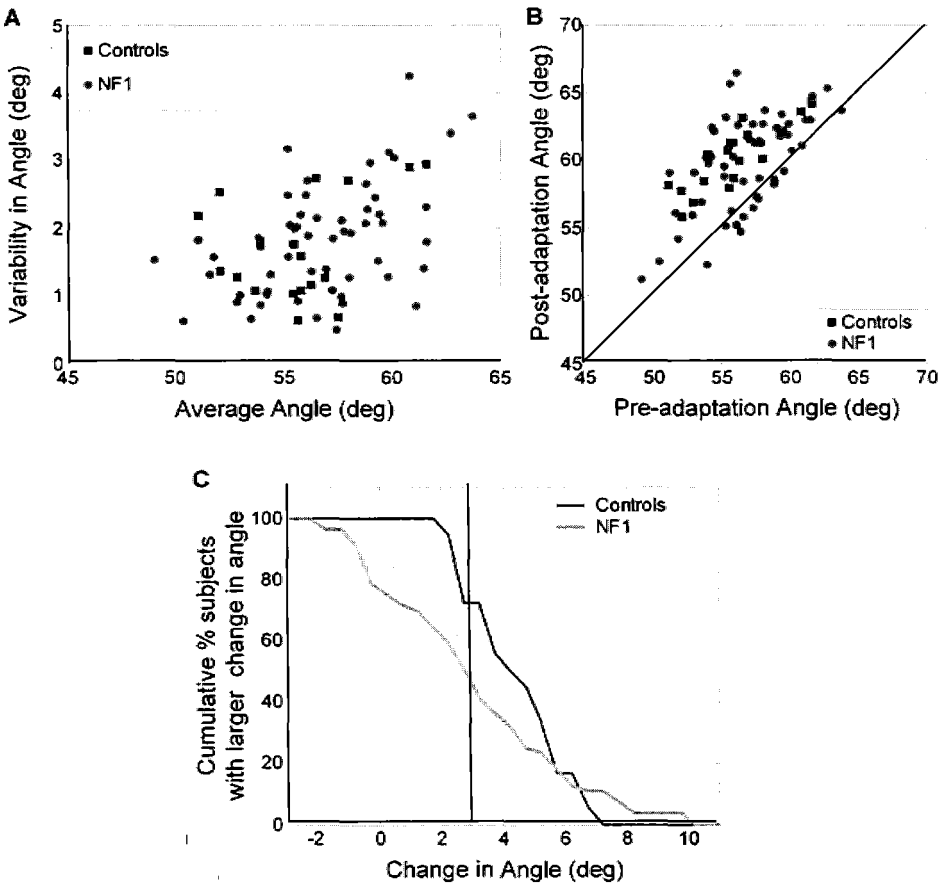

Figure 3: Prism adaptation

Panel A shows the variability versus the average hand movement angle of 56 NF1 children and 18 healthy controls in the pre-adaptation condition (without visual feedback of the hand); each dot represents one individual subject. Panel B shows the average angle of the arm movements in the post-adaptation phase versus the average angle of the arm movements in the pre-adaptation phase for these children; the oblique line is the unity line. Panel C shows the cumulative distribution of the changes in average movement angles between the pre- and post adaptation phases in the NF1 and the control group. The vertical line indicates the cut-off point for significant prism adaptation (2.93 degrees).

After wearing prism goggles with visual feedback in the adaptation phase, the average movements in the post-adaptation phase did not differ between the groups (movement angle:

59.9 ± 3.6 degrees in NF1 vs. 60.1 ± 2.3 degrees in controls, p=0.8; distance: 24.0 ± 2.3 cm in NF1 vs. 23.7 ± 1.9 cm in controls, p=0.7). However, the changes in movement angle between the pre-adaptation and post-adaptation phase induced by the prism goggles was significantly smaller in NF1 children than in controls (3.1 ± 3.0 vs. 4.5 ± 1.6 degrees, respectively, p=0.03, see figure 3B).

As can be seen in figure 3B, some NF1 children did show a significant prism adaptation (Change in angle > 2.9 degrees, as derived from the control group, with $p<0.01$). However, the proportion of subjects with a significant adaptation tended to be smaller in the NF1 group (28 out of 56 NF1 children (50%)) than in the control group (13 out of 18 controls (72%), $\chi^2=2.72$, p=0.1). The difference in distributions of the changes in hand movement angles between the two groups was marginally significant (absolute extreme difference = 0.375, Z=1.32, p=0.06, see figure 3C).

Age was not related to performance on prism adaptation in children with NF1 or controls (R=0.08, p=0.6 for NF1 and R= -0.32, p=0.2 for controls). We did not observe any correlations between Beery VMI scores and the outcomes of the motor tests in NF1 children and controls (all p>0.5).

Discussion

In the present study, motor performance and motor learning capacities of children with NF1 was assessed using the Beery VMI, saccade adaptation and prism adaptation tasks, and compared to healthy age-matched controls. As expected, children with NF1 show lower scores on the Beery VMI task. In addition, the adaptation of hand movements to prism goggles was reduced in NF1 children. However, saccadic performance and plasticity were not affected, as well as the performance of goal directed hand movements.

As expected, NF1 children scored significantly lower on the Beery VMI task than controls. We observed the typical visual-spatial problems, which are in line with the poorly developed visual-spatial skills of children with NF1 (reviewed by Ozonoff)[35], but we found that some copying errors were related to problems fine motor coordination. Impairments in the Beery VMI can indicate problems in a variety of brain areas, including the right hemisphere, the primary motor cortex of the dominant hand, the cerebellum, subcortical nuclei, and/or the corpus callosum.[5]

Baseline saccadic accuracy and the ability to adapt saccadic eye movements in NF1 appeared normal. In the present study, 55% of the NF1 and 64% of the control children were able to modify the amplitudes of their saccades in a classical saccade adaptation paradigm. These percentages are in good agreement with the 66% found in a group of 39 healthy children.[36] The saccadic oculomotor system comprises a network of brain areas, including the cerebellum and the parietal and frontal cortex, the basal ganglia, the superior colliculus and the brainstem.[37] The cerebellar oculomotor vermis is critically involved in maintaining a high saccadic accuracy.[19-21] Impairments in saccadic latencies and directions have been observed previously in a small group of 10 children with NF1, which were postulated to reflect involvement of a broad (cortical) network of brain areas involved in saccadic control.[38]. Our results suggest that the oculomotor vermis of the cerebellum is less likely to be part of that potentially deficient network in NF1.

Prism adaptation seems to be impaired in children with NF1. Although both groups show a significant change in the angle of the hand movements after prism glass displacement, the average degree of adaptation is significantly smaller in NF1 children. Furthermore, the variability in performance between NF1 children is larger than between control children and fewer NF1 children show a significant prism-induced after-effect. A subgroup of NF1 children could adapt their hand movements quite adequately, whereas others did not adapt at all. This seems to be in line with the large variability in clinical and cognitive characteristics between patients with NF1.[39] Impaired prism adaptation could result from problems in the anterior and caudal posterior lobe of the cerebellar cortex (including C1 – C3 zones), but also upon other motor areas such as the ventral premotor cortex and the posterior parietal cortex, which is involved in visually directed movements.[24, 40]

A potential limitation of our study is that a few NF1 children did not perform properly in the saccade and prism adaptation tasks, for instance by making large head movements, or by not making goal directed hand movements, which excluded them from further analysis. This was likely due to a short attention span or inability to understand the instructions completely. Although these tests have been administered successfully in children with and without mental retardation [28, 30, 36], it could be that the attention and cognitive deficits in these excluded children is related to worse motor performance. Their exclusion may have led to an overestimation of the performance of NF1 children on this task.

Taken together, our findings suggest that the motor problems displayed by children with NF1 may partially be related to deficits in plasticity of motor control.[41] However, our results suggest

that these deficits do not arise from a single brain region as a whole, such as the cerebellum. Rather, specific parts of the cerebellum and cerebrum may be selectively affected. Future research is necessary to unravel the neuronal basis of motor problems in patients with NF1.

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Documentation of Author Roles

This study was conceived by JNvdG, CIdZ, and YE, and funding was obtained by CIdZ and YE. The study was organized by LCK, JNvdG, AdGB, HAM, and YE. Data collection was performed by LCK, AdGB, FKA, and JNvdG. Statistical analyses were designed and executed by LCK, JNvdG and FKA, and, in addition, reviewed by YE. The first draft of the manuscript was written by LCK and JNvdG, and critically reviewed by all authors.

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CHAPTER 6

Quantitative differentiation between healthy and disordered brain matter in Neurofibromatosis type I patients using Diffusion Tensor Imaging

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Abstract

Background and Purpose:

Hyperintensities on T2-weighted images are seen in the brains of most patients with Neurofibromatosis Type I (NF1), but the origin of these unidentified bright objects (UBOs) remains obscure. In the current study, we examined the diffusion characteristics of brain tissue in children with NFI to test the hypothesis that a microstructural abnormality is present in NF1.

Materials and Methods:

Diffusion tensor imaging (DTI) was performed in 50 children with NF1 and 8 controls. Circular regions of interest were manually placed in 7 standardized locations in both hemispheres, including UBO sites. Apparent diffusion coefficients (ADC), fractional anisotropy (FA) and axial anisotropy (Am) were used to differentiate quantitatively between healthy and disordered brain matter. Differences in eigenvalues ($\lambda 1$, $\lambda 2$, $\lambda 3$) were determined to examine parenchymal integrity.

Results:

We found higher ADC values for UBOs than for normal-appearing sites ($p < 0.01$), and higher ADC values for normal-appearing sites than for controls ($p < 0.04$ in 5 of 7 regions). In most regions, we found no differences in FA or A_m. Eigenvalues $\lambda 2$ and $\lambda 3$ were higher at UBO sites than in normal-appearing sites ($p < 0.04$).

Conclusion:

With ADC, it was possible to differentiate quantitatively between normal- and abnormal-appearing brain matter in NF1 and also between normal appearing brain matter in NF1 and healthy brain matter in controls, indicating subtle pathologic damage, disrupting the tissue microstructure in the NF1 brain. Higher diffusivity for $\lambda 1$, $\lambda 2$ and $\lambda 3$ indicates that this disturbance of microstructure is caused by accumulation of fluid or vacuolation.

Introduction

Hyperintensities on T2-weighted images are seen in the brains of most patients with Neurofibromatosis Type I (NF1). Although many imaging techniques have been used to assess these unidentified bright objects (UBOs), their origin remains obscure.[1-4] The only pathologic study performed in NF1 so far revealed intramyelinic vacuolar changes or spongiotic myelinopathy that correlated with the hyperintensities found on T2-weighted images.[5] In addition to conventional MR imaging, several studies have used diffusion-weighted-imaging (DWI) with assessment of apparent diffusion coefficients (ADCs), to gain information on UBOs that cannot be assessed by inspection of conventional images alone. On the basis of high ADC values, a widespread myelin disorder was suggested to be present in patients with NF1.[6-9] However, ADC reflects only the magnitude of the diffusion of water molecules. Although high ADC values might suggest increased water content of the brain, with ADC alone, it is not possible to examine the microstructural integrity of the parenchyma. Diffusion tensor imaging (DTI), which measures the degree and direction of molecular diffusivity, is able to detect white matter abnormalities and characterize them in terms of white matter fiber integrity.[10, 11]

DTI generates a diffusion tensor matrix from a series of DWIs. By matrix diagonalization, the 3 eigenvalues $\lambda 1$, $\lambda 2$ and $\lambda 3$ can be calculated. $\lambda 1$ has the largest value and reflects the diffusivity parallel to a structure; $\lambda 2$ and $\lambda 3$ are the middle and smallest eigenvalues, respectively; and their average represents the diffusivity perpendicular to a structure. Various anisotropy indexes (fractional anisotropy [FA], axial inisotropy [A_m]) can be calculated by using the eigenvalues. They describe the ratio of the eigenvalues and are scaled from zero (isotropic) to 1 (anisotropic) and reflect the microstructure of white matter tracts. Looking at the eigenvalues themselves enables specific assessment of myelin integrity, as distinct from axonal integrity.[12, 13] Recently, a DTI study on adult patients with NF1 revealed higher ADC and lower FA values. Unfortunately, changes in eigenvalues were not reported.[14]

The purpose of this study was to examine the diffusion characteristics of brain tissue in children with NF1 by means of DTI and to test the hypothesis that a microstructural abnormality is present in NF1. We tested this in three ways: First, by assessing ADC and indexes of anisotropic diffusion, we tried to differentiate quantitatively between normal- and abnormal-appearing brain tissue in children with NF1. Second, we examined the normal-appearing parenchyma in NF1 to see if it is different from parenchyma in healthy controls. Third, we looked at parenchymal integrity at UBO sites and normal appearing sites by assessment of the eigenvalues. In addition,

because T2-weighted hyperintensities in the hippocampus have been suggested to have a different pathogenetic basis from classic UBOs,[15] we paid special attention to the diffusion characteristics of the hippocampal hyperintensities to see if they were different from those in other regions of interest.

Material and Methods

Subjects

Data for this study were obtained in the context of a larger study on NF1 and cognitive functioning. All participants were recruited from the multidisciplinary NF1 outpatient clinic of the hospital. Inclusion criteria were the following: age 8 to 17 years, NF1 diagnosis according to the criteria of the National Institutes of Health[16] and informed consent from parents and children older than 12 years of age. Exclusion criteria were: segmental NF1 (because brain involvement is not certain in these patients), pathology of the central nervous system (CNS) (other than asymptomatic gliomas), deafness, severe impaired vision, use of antiepileptics, inefficient production and comprehension of the Dutch language, and severe mental retardation (intelligence quotient <48).

One hundred twenty-six Children fulfilled age criterion. Twelve children were excluded on the basis of possible segmental NF1 (n = 3), use of antiepileptics (n = 3), hydrocephalus (n = 3), severe mental retardation (n = 1) and inefficient production and comprehension of the Dutch language (n = 2). The remaining 114 children were invited to participate in the larger study, of which 62 consented. The study was approved by the medical ethics committee of our institution. A total of 50 out of 62 children that participated in the larger study consented to MR imaging (21 girls, mean age 12.2 years; range: 8.1-15.7 years, and 29 boys, mean age 12.3 years; range: 8.0-16.5 years).

Image acquisition

MR anatomic imaging with DTI was performed using a 1.5T system (EchoSpeed; GE Healthcare, Milwaukee, Wis) and a dedicated 8-channel head coil. DTI data were acquired by using a multi-repetition single-shot echo-planar sequence with a section thickness of 3 mm with no gap. The DTI images were obtained in 25 gradient directions with a sensitivity of b = 1000 s/mm², TR = 15000 ms, TE = 82.1 ms, 1 average, FOV of 240 x 240 mm², a matrix of 128 x 128 resulting in a voxel size of 1.8 x 1.8 x 3.0 mm3. Acquisition time was 5:28 minutes with a total of 53 sections to cover the entire brain.

Data collection

All images were analyzed by visual inspection by an experienced pediatric neuroradiologist to exclude CNS tumors. Hyperintense lesions on T2-weighted, fluid-attenuated inversion recovery (FLAIR) and diffusion images (b = 0 s/mm²) were classified as UBOs, when no hyperintense lesion was present, the area was scored as normal-appearing site.

For quantitative data analysis, ADC, FA, A_m, and eigenvalues were used. FA measures the fraction of the magnitude of the diffusion tensor that can be ascribed to anisotropic diffusion.[17] Am reflects the shape of the diffusion ellipsoid.[18] ADC, FA, and eigenvalue maps were reconstructed by using commercially available software (Functool 3.1.23, Advanced Workstation 4.1; GE Healthcare). Circular regions of interest of specific sizes were manually placed in 7 predetermined anatomic locations in both hemispheres: the cerebral peduncle (CP), cerebellar white matter (CWM), hippocampus (HI), thalamus (TH), globus pallidus (GP), and frontal (FWM) and parieto-occipital (POWM) white matter. The size of the regions of interest was 100 mm² for CWM, 70 mm² for CP, 170 mm² for HI, POWM and FWM, and 130 mm² for GP and TH, according to the method of Alkan et al (Fig 1).[8] All regions of interest placement was done on the b = 0 s/mm² images because anatomic detail was better on these images than on the computed maps, ensuring anatomic precision. Regions of interest were automatically superimposed on the functional maps by the software that was used in this study. ADC maps were used to exclude CSF from measurements to minimize overestimation of the ADC values. FA maps were used when placing regions of interest in the GP and TH region to avoid as much as possible the involvement of the corticospinal tract (anterior and posterior limb of the internal capsula).

Statistical analysis

For statistical analysis ADC, FA, and eigenvalues calculated by the software were used. Am values were calculated using the eigenvalues λ1, λ2 and λ3 in the following way:

$$A_m = \frac{\lambda_1 - \frac{1}{2}(\lambda_2 + \lambda_3)}{\lambda_1 + \lambda_2 + \lambda_3}$$

To determine intra-observer reliability of region-of-interest placement, the same observer repeated the placement in a subset of 10 scans. Single measure intra class correlation coefficients (ICC) were calculated to compare the variability of data obtained.

To explore mean group differences, we averaged values obtained of the left and right hemisphere per region, per subject. All regions were then divided into the following groups based on NF1 and on the prevalence of UBOs: (1) NF1 regions with bilateral UBOs or with a UBO on 1 site and a normal-appearing contralateral site, (2) NF1 regions with no UBOs (both sites normal appearing); and (3) healthy controls. Differences in ADC, FA, Am, and eigenvalues were compared by using 1-way analysis of variance (ANOVA) or the Kruskall-Wallis test (if the distribution of the data was skewed). Significance was set at $p < 0.05$ and post hoc comparisons were done by using Scheffe. Differences in the proportions of $\lambda 1$ and $\lambda 2$, as well as the proportion of $\lambda 1$ and $\lambda 3$, between HI hyperintensities and UBOs in other regions, were analyzed using Wilcoxon signed ranks test for nonparametric related samples. Statistical analysis was done by using the Statistical Package for the Social Sciences 10.0 for Windows (SPSS, Chicago, Ill).

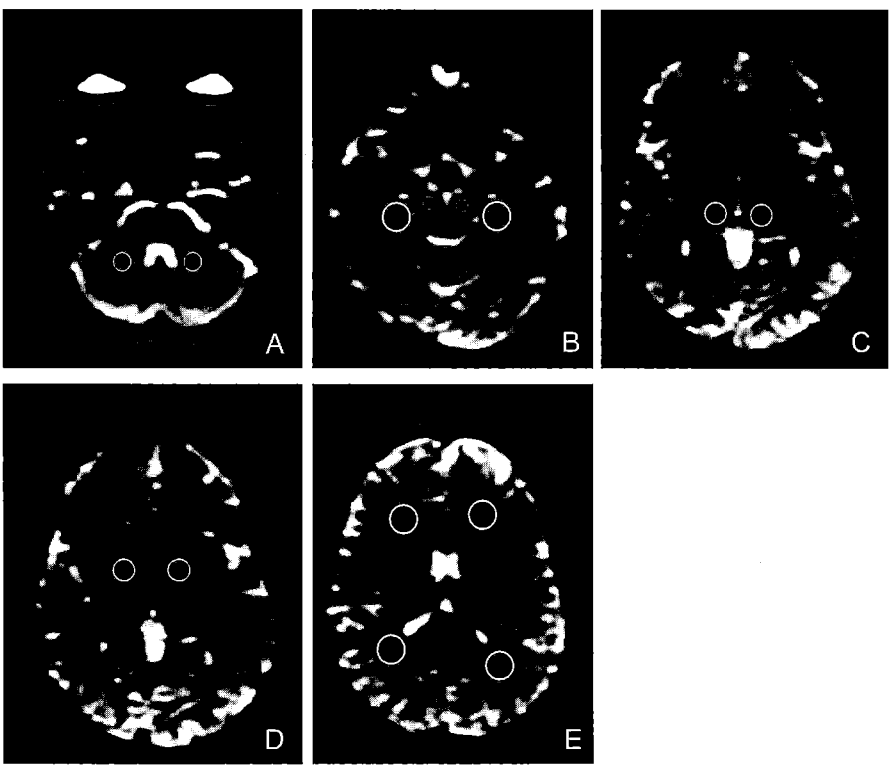

Figure 1: Region-of-interest placement.

Transverse b = 0 s/mm² images of a healthy control (multi repetition, single shot echo-planar sequence; slice thickness of 3 mm with no gap; 25 gradient directions; b = 1000 s/mm²; TR/TE = 15000/82.1 ms; 1 average; field of view of 240 x 240 mm²; matrix of 128 x 128; voxel size of 1.8 x 1.8 x 3.0 mm³). Circular Regions-of-Interest are placed in the (A) cerebellar white matter, (B) cerebral peduncle and hippocampus, (C) thalamus, (D) globus pallidus, and (E) frontal and parieto-occipital white matter.

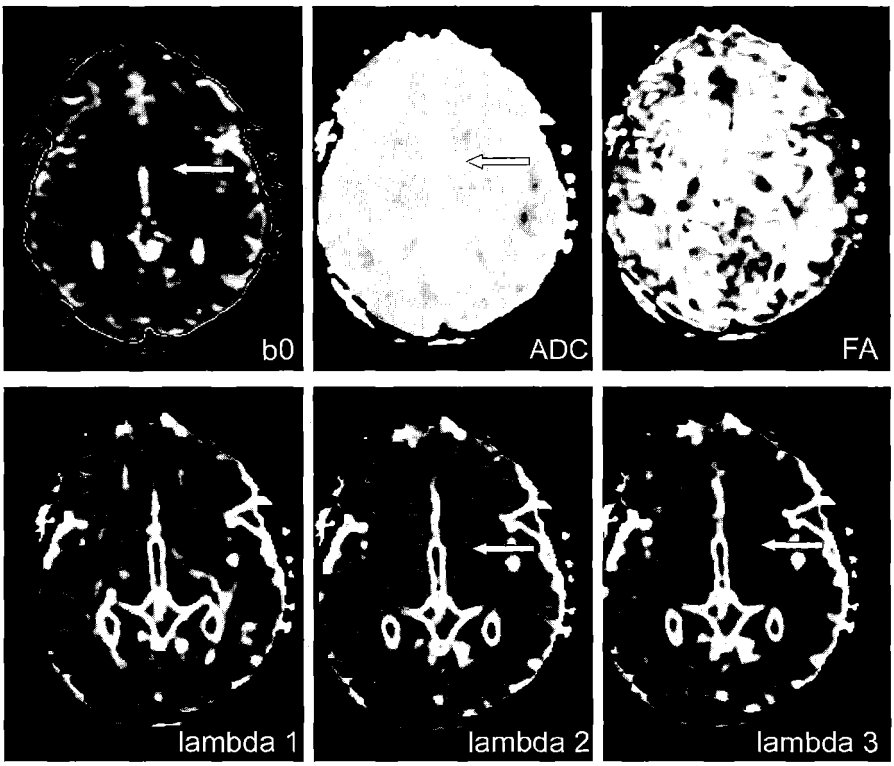

Figure 2: Diffusion Tensor Images of the globus pallidus.

Transverse Diffusion Tensor Images (multi repetition, single shot echo-planar sequence; slice thickness of 3 mm with no gap; 25 gradient directions; b = 1000 s/mm^2; TR/TE = 15000/82.1 ms; one average; field of view of 240 x 240 mm^2; matrix of 128 x 128; voxel size of 1.8 x 1.8 x 3.0 mm^3). Girl with NF1 (age 13 years) with an unilateral UBO in the Globus Pallidus. Arrow indicates an area of high intensity on the b = 0 mm^2/s image, and high values on the ADC-, λ_2- and λ_3-maps.

Results

Intra-observer reliability of region-of-interest placement

Reproducibility of region-of-interest placement by a single observer proved moderate to very good, with ICCs between 0.47 and 0.91. The lowest values were found for the GP (left hemisphere, 0.47; right hemisphere, 0.70), HI (left, 0.66; right, 0.55), and TH (left, 0.52; right, 0.73), whereas the highest ICCs were found for CWM (left, 0.67; right, 0.91). However, the wide range of ICCs in these regions is disconcerting. In FWM, POWM and CP the ICC was >0.71.

Qualitative evaluation of UBOs

Controls (4 girls, age range: 7.4-12.1 years, mean age 10.8 years and 4 boys, age range: 7.8-12.0 years, mean age 9.6 years) were selected for comparison. All controls were without chronic disease and had normal findings on MR imaging.

Sixty-eight percent of the children with NF1 (n = 34) were found to have UBOs in 1 or more of the 7 selected regions, which could be detected and measured on the b = 0 s/mm² images. Forty-six percent of the children with NF1 had UBOs in the GP (13 bilateral, 10 unilateral), 14% in the TH (2 bilateral, 5 unilateral), 50% in the CWM (16 bilateral, 9 unilateral) and 22% in the CP (9 bilateral, 7 unilateral). The HI was visually scored bilaterally hyperintense on T2- and FLAIR images in 48% of the children with NF1. No circumscript UBOs were found in POWM and FWM. **Figure 2** presents ADC, FA, and eigenvalues maps of a girl with NF1 with a UBO in the left GP and a normal-appearing contralateral side.

Measurements in the CP could not be performed in 2 children with NF1, and in 1 of those children, motion artefacts also prohibited measurements in the HI. Means ± SD of ADC, FA, A_m, and eigenvalues per region per group are plotted in **Figures 3-6.**

Quantitative differentiation of healthy and disordered brain matter in NF1

ADC values were significantly higher in regions where UBOs were present than in the matching regions with no UBOs (for all regions, $p < 0.01$). Also, ADC values were higher in NF1 regions with no UBOs than in the regions of healthy controls, significantly so in the POWM and CWM ($p < 0.03$), FWM ($p < 0.01$), GP ($p < 0.04$) and TH ($p < 0.01$; **Figure 3**).

To investigate the influence of region-of-interest size, we performed additional measurements in the GP region in a subset of 15 patients, by using a circular region-of-interest of 30 mm². This smaller region-of-interest size resulted in a significantly higher ADC value ($p < 0.01$) as compared with the value obtained by a region-of-interest of 130 mm².

With respect to FA values, the results were not as clear-cut. Although there was a trend toward FA values in NF1 regions with UBOs being lower than those in matching NF1 regions without UBOs and controls, these differences were not significant for most regions **(Figure 4).** Only for CWM was the FA value in NF1 with UBOs significantly lower than that in NF1 without UBOs ($p < 0.01$). Remarkably, results in the GP were opposite to those in other tissues, with higher FA values for NF1 with UBOs as compared to NF1 without UBOs ($p < 0.01$).

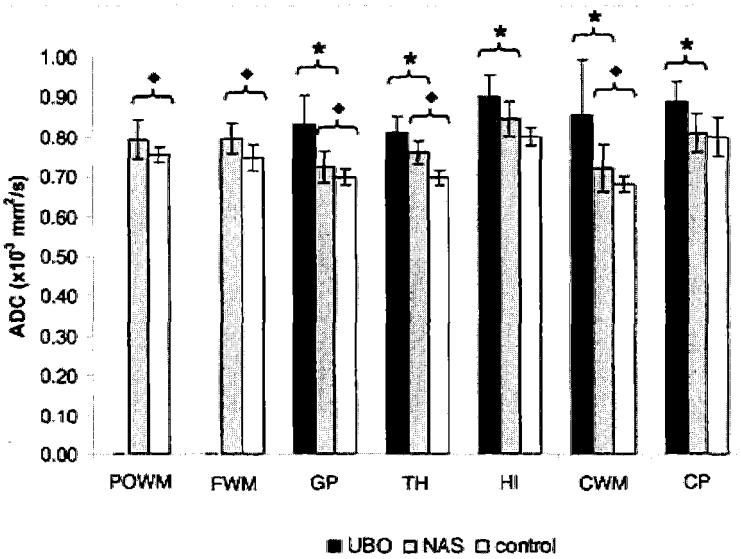

Figure 3: Mean ADC values. The cohort is divided in three groups: NF1 regions with UBOs (UBO), NF1 regions with normal-appearing brain matter (NAS) and controls. Mean ADC values for UBOs are higher than those for NAS in all regions (except POWM and FWM were no UBOs were found). Mean ADC values for NAS are significantly higher than those of controls in all regions, except CP. For convenience, the results of the hippocampal area are presented in the same figure as the results of the other regions-of-interest. Hippocampal hyperintensities are presented as UBO and normal appearing hippocampal areas as NAS. Significant differences between UBO and NAS are indicated by ✱, between NAS and controls by ◆. CP: cerebral peduncle, CWM: cerebellar white matter, HI: hippocampus, TH: thalamus, GP: globus pallidus, FWM: frontal white matter, and POWM: parieto-occipital white matter

A_m values were lower in NF1 regions with UBOs as compared with NF1 regions without UBOs, significantly so in CWM ($p < 0.02$) and CP ($p < 0.01$). In addition, A_m values were also significantly lower in NF1 regions without UBOs as compared with controls in the TH ($p < 0.02$). In GP, an opposite trend was shown: the value found for NF1 with UBOs was significantly higher as compared with that of NF1 without UBOs ($p < 0.01$). No significant differences in A_m were found between UBO, normal-appearing sites, and controls in the other regions assessed. **(Figure 5).**

Microstructural integrity of NF1 brains

To examine microstructural integrity of the brain parenchyma in NF1, we assessed the eigenvalues **(table 1)**. In all regions tested, NF1 regions with UBOs had eigenvalues higher than those of NF1 regions without UBOs **(Figure 6)**. In CWM and GP all three eigenvalues

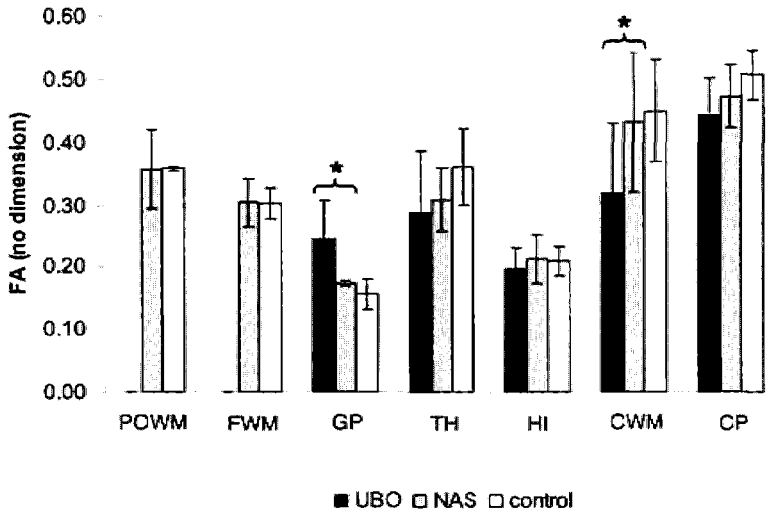

Figure 4: FA values.

Mean FA values ± SD for UBO, normal-appearing site (NAS) and controls are plotted for the 7 regions of interest. Few significant differences are found between UBO and NAS indicated by ✱. CP: cerebral peduncle, CWM: cerebellar white matter, HI: hippocampus, TH: thalamus, GP: globus pallidus, FWM: frontal-, and POWM: parieto-occipital white matter.

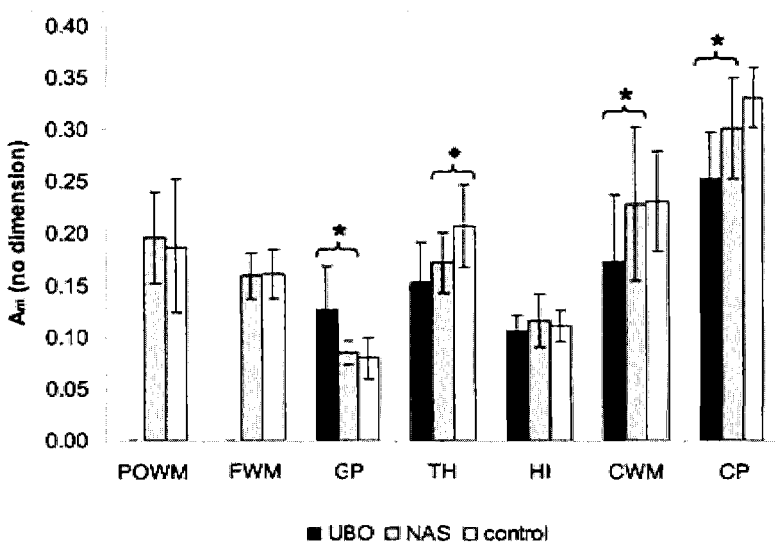

Figure 5: A_m values.

Mean A_m values ± SD for UBO, normal-appearing site (NAS) and controls per regions of interest. Significant differences between UBO and NAS are indicated by ✱ and between NAS and controls by ◆. CP: cerebral peduncle, CWM: cerebellar white matter, HI: hippocampus, TH: thalamus, GP: globus pallidus, FWM: frontal-, and POWM: parieto-occipital white matter.

were significantly higher ($p < 0.02$), in HI, CP and TH, λ_2 and λ_3 were significantly higher ($p < 0.04$), indicating a loss of microstructure of the brain parenchyma in children with NF1 with UBOs.

The eigenvalues in NF1 regions without UBOs closely followed those in healthy controls, indicating that the microstructure is close to normal in children without UBOs, even though some slight but significant elevations were seen (FWM, $p < 0.01$ for λ_1 and λ_2; HI, $p < 0.02$ for λ_1). The exception is in TH, where the eigenvalues of NF1 without UBOs are much higher than those of healthy controls, significantly so for λ_2 and λ_3 ($p < 0.01$).

Table 1: DTI values per ROI)*

ROI	Group	N	Mean Value					
			ADC x 10^{-3} mm^2/s	FA	λ_1 x 10^{-3} mm^2/s	λ_2 x 10^{-3} mm^2/s	λ_3 x 10^{-3} mm^2/s	A_m
POWM	NAS	50	0.79 ± 0.05	0.31 ± 0.06	1.09 ± 0.06	0.74 ± 0.06	0.52 ± 0.07	0.20 ± 0.04
	Controls	8	0.75 ± 0.02	0.36 ± 0.06	1.06 ± 0.04	0.70 ± 0.05	0.48 ± 0.03	0.19 ± 0.06
FWM	NAS	50	0.80 ± 0.04	0.31 ± 0.04	1.04 ± 0.05	0.76 ± 0.04	0.56 ± 0.05	0.16 ± 0.02
	Controls	8	0.75 ± 0.03	0.30 ± 0.02	0.98 ± 0.04	0.71 ± 0.04	0.53 ± 0.04	0.16 ± 0.02
GP†	UBO	23	0.83 ± 0.07	0.25 ± 0.06	1.02 ± 0.12	0.79 ± 0.07	0.64 ± 0.06	0.13 ± 0.04
	NAS	27	0.72 ± 0.04	0.17 ± 0.04	0.83 ± 0.05	0.70 ± 0.04	0.60 ± 0.04	0.09 ± 0.01
	Controls	8	0.70 ± 0.02	0.16 ± 0.02	0.81 ± 0.03	0.68 ± 0.03	0.58 ± 0.03	0.23 ± 0.02
TH	UBO	7	0.81 ± 0.04	0.29 ± 0.10	1.04 ± 0.05	0.75 ± 0.06	0.61 ± 0.08	0.15 ± 0.04
	NAS	43	0.76 ± 0.03	0.31 ± 0.05	1.02 ± 0.05	0.70 ± 0.04	0.55 ± 0.04	0.17 ± 0.03
	Controls	8	0.70 ± 0.02	0.36 ± 0.06	0.98 ± 0.07	0.63 ± 0.03	0.47 ± 0.02	0.21 ± 0.04
HI	UBO	23	0.90 ± 0.05	0.20 ± 0.04	1.07 ± 0.51	0.87 ± 0.42	0.72 ± 0.51	0.10 ± 0.03
	NAS	25	0.84 ± 0.04	0.21 ± 0.04	1.04 ± 0.07	0.82 ± 0.04	0.66 ± 0.04	0.12 ± 0.03
	Controls	8	0.80 ± 0.02	0.21 ± 0.02	0.97 ± 0.02	0.78 ± 0.03	0.63 ± 0.03	0.11 ± 0.02
CWM†	UBO	25	0.85 ± 0.14	0.32 ± 0.11	1.11 ± 0.08	0.85 ± 0.35	0.61 ± 0.13	0.20 ± 0.18
	NAS	25	0.72 ± 0.06	0.43 ± 0.11	1.05 ± 0.13	0.65 ± 0.07	0.47 ± 0.09	0.23 ± 0.07
	Controls	8	0.68 ± 0.02	0.45 ± 0.08	1.00 ± 0.06	0.62 ± 0.05	0.43 ± 0.04	0.23 ± 0.05
CP	UBO	16	0.89 ± 0.05	0.44 ± 0.06	1.31 ± 0.09	0.77 ± 0.09	0.54 ± 0.09	0.25 ± 0.04
	NAS	33	0.81 ± 0.05	0.47 ± 0.05	1.32 ± 0.11	0.69 ± 0.09	0.47 ± 0.05	0.30 ± 0.05
	Controls	8	0.80 ± 0.05	0.51 ± 0.04	1.32 ± 0.12	0.63 ± 0.05	0.43 ± 0.04	0.33 ± 0.03

ROI indicates region of interest; NAS, normal-appearing sites; POWM, parieto-occipital white matter; FWM, frontal white matter; GP, globus pallidus; TH, thalamus; HI, hippocampus; CWM, cerebellar white matter; CP, cerebral peduncle; UBO, unidentified bright object; ADC, apparent diffusion coefficient; FA, fractional anisotropy; A_m, axial anisotropy.

* Values are averaged for left and right hemispheres. For all indices, mean values ± SD are given for regions with UBOs, regions without UBOs (NAS), and healthy controls.

† Nonparametric data.

Analysis of the difference in proportion of λ_1 and λ_2 for hyperintensities in the HI and for UBOs in other regions revealed there were no differences in the proportion of λ_1-λ_2 for HI compared with the TH ($p < 0.11$) or GP ($p < 0.59$). The same results were found for the

differences in the proportions of λ_1 and λ_3. This indicates that the diffusion perpendicular to the axon for HI is not different from other grey matter areas like TH and GP. Compared with white matter areas CP and CWM, there were significantly different proportions for λ_1-λ_2 ($p < 0.02$ and $p < 0.01$).

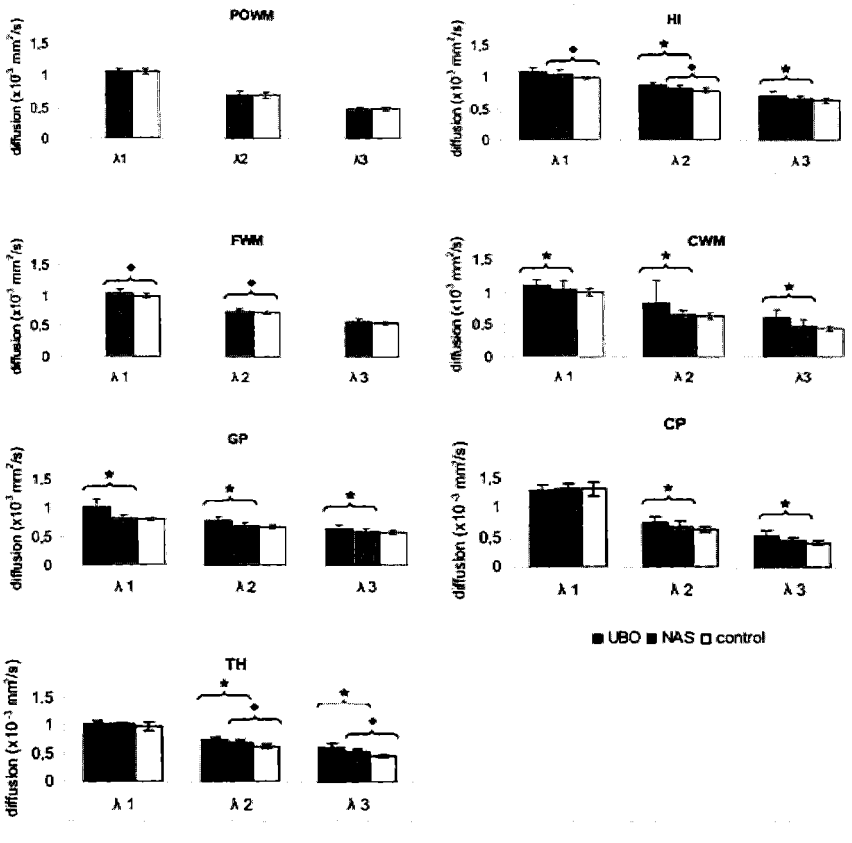

Figure 6: Eigenvalues per region.

For UBO, normal-appearing sites (NAS) and controls, mean values for the three eigenvalues are plotted for each region. In all regions were UBOs are found, λ1, λ2 and λ3 are higher for UBOs than NAS and higher for NAS compared with controls. Significant differences between UBO and NAS are indicated by ✱ and between NAS and controls by ◆.

Discussion

DTI in 50 Children with NF1 and 8 controls revealed significantly higher ADC values in NF1 regions with UBOs as compared to NF1 regions without UBOs and in NF1 regions without

UBOs as compared to controls. ADC values reflect the overall brain water content,[19] thus the differences between NF1 regions with UBOs, NF1 regions without UBOs and controls found in this study can primarily be explained by increased water content of the brain parenchyma in NF1, which is apparently exacerbated in NF1 regions with UBOs. These findings confirm previous reports.[6-9, 14] However, the ADC values found in this study are not as high as the ADC values found previously. An explanation of lower ADC values may be found in the shorter effective TE and different b-value that was used in our scan protocol compared to reported DWI protocols.[20] Also DTI improves the ability to avoid partial volume effect of CSF by fine tuning region-of-interest placement by simultaneous use of b = 0 s/mm² images, ADC, and FA maps. Another important technical issue is the chosen size of the region-of-interest, since it influences the ADC values. Larger regions of interest result in lower ADC values because the value represents an average of more voxels, the area of the UBO and surrounding tissue, as demonstrated in the GP by the subtest in which a region-of-interest of 30 mm² instead of 130 mm² was used.

DTI may facilitate a better understanding of the abnormalities seen in NF1 because evaluation of microstructural integrity of the parenchyma can be achieved by assessing anisotropy indexes and eigenvalues. Anisotropy can be influenced by factors such as axon packing, relative membrane permeability to water, internal axon structure, myelination, and tissue water content.[21] ADC values in this study were higher in NF1 regions with UBOs and in NF1 regions without UBOs than in healthy controls, suggesting increased tissue water content or decreased axon packing. However, based on FA values in our study, it was only possible to differentiate between NF1 regions with UBOs and NF1 regions without UBOs in the CWM and GP. Remarkably, in the latter, we found higher FA values for NF1 with UBOs as compared to NF1 without UBOs and controls, which is a counterintuitive finding.

When carefully re-examining region-of-interest placements in the GP, it became clear that it is almost impossible to avoid partial volume effects of the posterior limb internal capsula, even when regions-of-interest were drawn smaller (30 mm² instead of 130 mm²). UBOs are typically found very near or in some cases *in* the internal capsula. The high anisotropy of the internal capsula affects the measured FA values for the GP. Low ICC and high variability (Fig 3) also show the difficulties of taking measurements in the GP-region. In contrast to our results in children with NF1, a recent published study on adult NF1 patients using DTI found significantly lower FA values in NF1 brains than in healthy brains, indicating generalized microstructural alterations and dysmyelination in adult patients with NF1.[14] A caveat of the

study in adult patients is that it did not assess alterations of the eigenvalues, and it is, therefore, not possible to relate changes in FA to dysmyelination. Lower FA values at UBO-sites might also be caused by damage to the axon as shown by MR Spectroscopy.[22] The study in adult patients found severely reduced concentrations of *N*-acetylaspartate at UBO sites, indicating that an increased myelin turnover was present, which could lead to subsequent axonal damage in adult NF1 patients. We found no evidence for axonal damage in children with NF1, which might be an explanation of why we did not find lowered FA values in our study.

We also did not find differences in the shape of the diffusion ellipsoid, when looking at A_m values, between children with NF1 with or without UBOs or healthy controls in most regions-of-interest. Although of all anisotropy indexes, A_m shows the strongest trend in relative changes,[23] in this study, A_m was only slightly more sensitive than FA. The homogenous attenuated white matter structures that in CWM and CP in contrast to GP, TH and HI, where the tissue also contains grey matter which has zero anisotropy,[24] could be the reason why we did find differences in anisotropy in CWM and CP between NF1 with UBOs and NF1 without UBOs, but not in the other regions-of-interest. If the eigenvalues λ_1 and λ_2 and/or λ_3 changed in the same direction, as found in our study, no differences in FA and A_m would be observed (but the change in ADC would be marked).[25]

Higher eigenvalues in NF1 regions with UBOs indicate that the microstructure of the parenchyma is different from the parenchyma in NF1 regions without UBOs. Animal studies have shown that an increment of axial diffusivity (λ_2 and λ_3) is related to myelin deficiency, whereas a decrease of parallel diffusivity (λ_1) indicates axonal disturbance.[26-28] Our findings of higher values for λ_2 and λ_3 indicate that diffusion perpendicular to the white matter structure is higher. Because we did not find lower values for λ_1 in NF1 regions with UBOs than in NF1 regions without UBOs, we hypothesize that the observed changes of the brain tissue in NF1 are not caused by damage to the axon, but relate to myelin deficiency. The higher value for *all* eigenvalues in our study, especially for λ_1, has not previously been reported in human or animal studies. Accumulation of fluid hypothetically should increase the magnitude of all three eigenvalues from their normal values.[29] Our study, therefore, indicates intramyelinic edema or vacuolar changes in the myelin. This confirms the study of DiPaolo et al,[5] which was the only pathologic study performed in NF1. Autopsy and histopathologic examination performed on 3 patients with NF1revealed intramyelinic vacuolar changes or spongiotic myelinopathy that correlated with the hyperintensities found on T2-weighted images. No stainable material was found within the vacuoles, which suggests that in life, they are filled with water.[5]

Notably, our study is not a radiology-pathology correlation study. To our knowledge, the specificity of changes of eigenvalues due to myelin disturbance in humans has not been tested. Because we were not able to perform histopathological examination in our cohort, no proof can be provided for the suggestion of myelin deficiency or vacuolar changes, however likely that eigenvalues will allow an assessment of myelin.

HI hyperintensities were of special interest in our study, since a different pathogenetic basis from classical UBOs was suggested.[15] Impairments in learning and behavior in mouse models of NF1 are thought to be suggestive of disordered hippocampal functioning.[15] We measured DTI parameters to examine if there is an underlying microstructural change in the hippocampal region distinct from other regions-of-interest. We found higher ADC values in hyperintense-appearing hippocampal areas than in normal-appearing hippocampal areas, which could explain disordered hippocampal functioning, but no differences in FA and A_m. Although anisotropy indices in HI were lower compared to other regions-of-interest, eigenvalues showed no different pattern when comparing HI to other grey matter structures like GP and TH. Therefore, distinct pathogenesis between hyperintense HI and classical UBOs cannot be concluded in this study by using DTI-parameters.

Our study has several limitations such as limited number of Children with NF1 with and without UBOs and even smaller number of control subjects. However, post-hoc power analyses showed that power was > 0.80 for all analyses in which NF-1 children with and without UBOs were compared with healthy controls for ADC values. Although our findings contribute to the unraveling of UBOs, for we have been able to prove that the high ADC values in UBOs observed in previous publications are due to increased axial diffusivity, no histological correlation with the observed diffusion signal abnormalities in NF1 could be provided.

Conclusion

Based on the results obtained in the current study, it can be concluded that it is possible to differentiate quantitatively between healthy and disordered brain parenchyma in children with NF1 using ADC values. Although no differences were found in anisotropy indexes, higher values for λ_2 and λ_3 in NF1 regions with UBOs than in NF1 regions without UBOs indicate higher axial diffusivity because of less obstruction (presumably due to water accumulation in myelin). The higher λ_1 contradicts axonal disturbance. The observed high diffusivity for all 3 eigenvalues (λ_1, λ_2 and λ_3) in NF1 regions with UBOs, as compared to NF1 regions without

UBOs and controls, supports pathological findings, and could indicate a disturbed microstructure of the NF1-brain due to accumulation of fluid or vacuolation when UBOs are present. A distinct pathogenesis between hyperintense HI and classic UBOs was not found in this study using by DTI-parameters.

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CHAPTER 7

The NF1 Simvastatin Trial

I

CHAPTER 7.1

The Effect of Simvastatin on Cognitive Functioning in Children With Neurofibromatosis Type 1: a Randomized Controlled Trial

I

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Abstract

Context

Neurofibromatosis type 1 (NF1) is among the most common genetic disorders that cause learning disabilities. Recently, it was shown that statin-mediated inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase restores the cognitive deficits in an NF1 mouse model.

Objective

To determine the effect of simvastatin on neuropsychological, neurophysiological, and neuroradiological outcome measures in children with NF1.

Design, setting and participants

Sixty-two out of 114 eligible children (54%) with NF1 participated in a randomized, double-blind, placebo-controlled trial conducted between January 20, 2006, and February 8, 2007, at an NF1 referral center at a Dutch university hospital.

Intervention

Simvastatin or placebo treatment once daily for 12 weeks.

Main Outcome Measures

Primary outcomes were scores on a Rey complex figure test (delayed recall), cancellation test (speed), prism adaptation, and the mean brain apparent diffusion coefficient based on magnetic resonance imaging. Secondary outcome measures were scores on the cancellation test (standard deviation), Stroop color word test, block design, object assembly, Rey complex figure test (copy), Beery developmental test of visual-motor integration, and judgment of line orientation. Scores were corrected for baseline performance, age, and sex.

Results

No significant differences were observed between the simvastatin and placebo groups on any primary outcome measure: Rey complex figure test (ß=0.10, 95% confidence interval [CI]: -0.36 to 0.56); cancellation test [speed] (ß=-0.19, 95% CI: -0.67 to 0.29); prism adaptation (odds ratio=2.0; 95% CI 0.55 to 7.37) and mean brain apparent diffusion coefficient (ß=0.06, 95% CI: -0.07 to 0.20). In the secondary outcome measures, we found a significant improvement in the simvastatin group in object assembly scores (ß=0.54, 95% CI: 0.08 to 1.01), which was

specifically observed in children with poor baseline performance (ß=0.80, 95% CI: 0.29 to 1.30). Other secondary outcome measures revealed no significant effect of simvastatin treatment.

Conclusions

In this 12-week trial, simvastatin did not improve cognitive function in children with NF1.

Trial registration

Trial registration isrctn.org Identifier: ISRCTN14965707

Introduction

Neurofibromatosis type 1 (NF1) is a common autosomal-dominant genetic disorder (incidence 1:3000)[1] caused by a mutation in the gene encoding neurofibromin, a protein that activates the hydrolysis of RAS-bound guanosine triphosphate.[2] NF1 is characterized by various neurocutaneous manifestations, problems in fine and gross motor functioning[3], as well as the frequent occurrence of cognitive disabilities. Children with NF1 have a lowered mean IQ (86-94), with particular deficits in visual-spatial skills, nonverbal long-term memory, executive functions and attention.[4-7] These problems have a large impact on school performance of children with NF1.[4] It has been suggested that the cognitive and motor deficits in children with NF1 are related to hyperintensities on T2-weighed magnetic resonance imaging of the brain[5, 8] that are characterized by high apparent diffusion coefficients (ADC values),[9] but some studies fail to confirm this relationship.[10]

Studies using mouse models for NF1 (*Nf1* mice) revealed that increased RAS/ERK signaling is primarily responsible for the neuronal plasticity deficits as well as the spatial learning and attention deficits of these mice.[11-13] RAS transforming activity requires isoprenylation (i.e., farnesylation or geranylgeranylation) of RAS, which can be blocked by farnesyl transferase inhibitors and by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA) reductase inhibitors.[14, 15] HMG-CoA reductase is the rate-limiting enzyme in the mevalonate pathway in which cholesterol and isoprenyl groups are synthesized. Importantly, treatment of *Nf1* mice with a farnesyl transferase inhibitor or HMG-CoA reductase inhibitor for just a few days, reverses the cognitive deficits of these mice.[11, 13] These findings are not only important for NF1, but also are of great interest for other neuro-cardio-facial-cutaneous syndromes (*e.g.* Noonan, Costello and cardio-facial-cutaneous syndromes), which are also caused by aberrant RAS/ERK

signaling, and for hamartoma syndromes (e.g., Cowden disease and tuberous sclerosis complex). The genes associated with these syndromes belong to a pathway that is not only coregulated by RAS but also critically dependent on RHEB, another farnesylated protein of the RAS family.

The favorable safety profile of the HMG-CoA reductase inhibitor simvastatin in adults and children[16] provided an opportunity to investigate whether the findings in the mouse model can be translated to humans. In a randomized, double-blind, placebo-controlled trial, we studied the effect of a 12-week simvastatin treatment on cognitive function of children with NF1 using neuropsychological, neurophysiological, and neuroradiological outcome measures.

Methods

Design

A prospective double-blind, placebo-controlled, randomized, single-site, 12-week clinical trial was conducted in children with NF1 between January 20, 2006, and February 8, 2007. The study was approved by the medical ethical committee of the Erasmus MC Rotterdam, the Netherlands.

Participants

All participants were recruited from the multidisciplinary NF1 outpatient clinic of the Erasmus MC – Sophia Children's Hospital, which is a university hospital and NF1 referral center in the Netherlands. Participants were enrolled by a pediatrician in the NF1 outpatient clinic (A.G.B.). Inclusion criteria were age 8 to 16 years, NF1 diagnosis according to the criteria of the National Institutes of Health,[17] and oral and written informed consent from parents and children older than 12 years. Exclusion criteria were segmental NF1, pathology of the central nervous system (other than asymptomatic gliomas), deafness, severely impaired vision, use of antiepileptic drugs, insufficient comprehension or use of the Dutch language, and an IQ below 48, which was assessed at baseline using the Wechsler Intelligence Scale for Children – Revised, Dutch version.[18]

Protocol

Patients were randomized to simvastatin or placebo using a permuted-block, 1:1 randomization list generated by the trial statistician (S.M.F.P.) with blocks of 6 participants, in which medication numbers 1 through 62 corresponded to either simvastatin or placebo. Randomization was performed by the Erasmus MC trial pharmacist who assigned patients a

medication number in the order of their enrollment in the trial and who dispensed the medication. Patients and all other investigators were blind to the treatment allocation. Patients were treated once a day in the morning for 12 weeks with simvastatin (weeks 0-4, 10 mg/d, weeks 5-8 20 mg/d, and weeks 9-12, 20 mg/d for children aged 8-12 years or 40 mg/d [taken as 2 20-mg doses] for children aged 13-16 years) or equivalent placebo. The placebo capsules were filled with microcrystalline cellulose PH102 and treatment capsules with a filler and a tablet of 10-mg (weeks 0-4) or 20-mg (weeks 5-12) simvastatin (film-coated; Alpharma Inc; Bridgewater, New Jersey). The capsules containing placebo or simvastatin were non-transparent and identical in color, shape, and size. Patients were instructed not to open the capsules. Patients were judged adherent when they took at least 80% of their study medication during the intervention period of 12 weeks, which was assessed by counting returned capsules.

Outcome measures

Outcome measures were assessed at baseline and after 12 weeks of treatment. For the primary outcome measures, we chose 2 neuropsychological tests that were analogous to statin-responsive tests in *Nf1* mice (measuring visual spatial memory and attention). In addition, we selected a neurophysiological and neuroradiological measure because we reasoned that these measurements would be insensitive to placebo or test-retest effects. This resulted in the following 4 primary outcome measures: performance on the Rey complex figure test (CFT) (delayed recall; assessing nonverbal long-term memory); performance on the cancellation test (speed, assessing attention), performance on a prism adaptation task (measurement of adaptation of the angle of hand movements in response to prism glass distortion,[19] which is thought to be dependent on cerebellar function[20, 21]), and mean apparent diffusion coefficient (ADC value) of the brain (mean ADC value of 7 predetermined anatomic locations predominantly affected by T2-weighed hyperintensities) as previously described.[9]

For the secondary outcome measures, we selected neuropsychological tests assessing domains that are specifically affected in patients with NF1: tests for attention and tests for visual-spatial skills with baseline scores of 1 SD or more below average.[4, 9] This resulted in the following secondary outcome measures: performance on the cancellation test (standard deviation; measuring attention fluctuations), the Stroop color word test, the block design test and object assembly test of the Wechsler Intelligence Scale for Children - Revised, the Rey CFT (copy), the Beery developmental test of visual-motor integration, and the judgment of line orientation task.[22]

Magnetic Resonance imaging was performed by using a 1.5-tesla system (EchoSpeed; GE Healthcare, Milwaukee, Wisconsin) and a dedicated 8-channel head coil. Diffusion tensor imaging data were gathered by using a multirepetition, singleshot echo-planar sequence with a section thickness of 3 mm with no gap. A 25-gradient directions technique was performed to obtain good diffusion tensor images (sensitivity, b=1000 s/mm2, repetition time 15000 ms; echo time, 82.1 milliseconds, 1 average; field of view, 240x240 mm2; matrix 128x128; voxel size, 1.8x1.8x3.0 mm^3) as described previously.[9]

All neuropsychological tests were developed for children, administered in their Dutch versions, and scored by 1 pediatric neuropsychologist (M.J.B.). Parallel versions of tests were applied when available to reduce the impact of practice effects. For technical reasons, left-handed children (n=7) were excluded from the prism adaptation task.

Treatment safety and adherence was assessed in the outpatient clinic at baseline, and after 4 and 12 weeks, and with a telephone consult after 8 weeks. Patients were provided with a diary in which they were instructed to note any deviations from treatment protocol and possible adverse events. At each consult, a general pediatrician recorded any adverse events and serious adverse events (adverse events that were life-threatening, causing disability, or requiring hospitalization) with a standardized checklist of the adverse events and serious adverse events reported with simvastatin use,[23] supported by open questions and a review of the patient's diary. All reported adverse events were scored as being not drug related, possibly drug related, or definitely drug related prior to unblinding. During the visits to the outpatient clinic, the pediatrician (A.G.B.) performed a standardized internal and neurological assessment, and blood was drawn for laboratory examination. We examined safety parameters alanine aminotransferase, aspartate aminotransferase and creatine phosphokinase, and efficacy parameters total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and triglycerides were examined according to standard clinical laboratory protocol. Criteria for discontinuation of study medication were a persistent of more than 3-fold the upper limit of normal (ULN) increases in alanine aminotransferase or aspartate aminotransferase levels, more than 10-fold the ULN for creatine phosphokinase with or without muscular symptoms, or 5- to 10-fold the ULN for creatine phosphokinase levels with muscular symptoms.[16]

Statistical analyses

One of the prominent effects seen in statin-treated *Nf1* mice was a recovery of their deficit in visual-spatial memory.[13] The Rey CFT (delayed recall) assesses the analogous domain of

nonverbal long-term memory in humans and has good psychometric properties, and performance on this test is specifically affected in patients with NF1.[24] Therefore, we based our power calculation on this test. On the assumption of a correlation of 0.70 between measurement before and after treatment, and a mean (SD) z-score of –1.32 (1.01) on the Rey CFT (delayed recall) at baseline,[24] we calculated that 30 persons were needed in both the placebo and treatment groups to ensure a power of 0.80 of detecting a significant (α=0.05) improvement in the Rey CFT (delayed recall) score up to -0.28 (difference of 1.04) in the treatment group.

All data were analyzed using SPSS 12.0 (SPSS Inc, Chicago, Illinois). For the neuropsychological tests, z-scores were used (with negative values indicating performance below the normative mean and positive values performance above the normative mean), except for the cancellation test (standard deviation) (raw score for non-normal distribution of reference values; larger negative values indicated larger attention fluctuations). Prism adaptation was scored to occur if the change (adaptation) of the angle of hand movements was significant ($p<0.01$) and larger than –1 SD of the mean change of age-matched healthy controls (n=16, unpublished observations). A decrease in ADC values indicates lower signal intensity.

Modified intention-to-treat analysis was performed for all patients with available 12-week test scores (n=61) without imputing missing values. Differences between the simvastatin and placebo groups at baseline were analyzed with the t-test, Mann-Whitney test, and χ^2 test. Differences between the simvastatin and placebo groups after 12 weeks of treatment were assessed using univariate and multivariate regression analysis. In the univariate analysis, we adjusted for baseline scores, and in the multivariate regression analysis we adjusted for baseline scores, age, and sex. Regression coefficients (ß) reflect the estimated differences in mean score at follow-up between the treatment groups with 95% confidence intervals (CIs). For categorical measures (prism adaptation), the difference between the treatment groups was expressed as an odds ratio with 95% CI. Cut-off level for significance was set at $p<0.05$. Effect modification of outcome parameters that were significantly different between the treatment and placebo groups after 12 weeks was examined using interaction terms between treatment and age and between treatment and baseline performance. The rationale for this were the conceivably higher brain plasticity in younger children and more room for improvement in children with low baseline performance, thus affecting the magnitude of response to simvastatin treatment. Subgroup analysis was performed only if effect modification was plausible ($p<0.10$ to take into account the small size of the subgroups) for addition of the interaction term to the multivariate analysis. All p-values reported are 2-sided. The outcome parameters and the method of statistical

analysis, including the subgroup analyses, were defined before unblinding. We did not correct for multiple comparisons for the following reasons. There are only 4 primary outcome measures, and they are specifically based on *a priori* assumptions. The outcome measures on the neuropsychological tests are potentially correlated, and correction would thus be inappropriate. By correcting for multiple comparisons, it would be very hard to detect a possible effect in a relatively small patient population. Thus we would run a high risk of discarding a promising drug while in fact there is an effect (Type II error).

For ethical reasons, an interim analysis was conducted by the statistician (S.M.F.P.) after 36 patients completed the study with complete maintenance of the double-blind protocol for all others. The criterion to discontinue the study was a significant difference between the simvastatin- and placebo groups on Rey CFT (delayed recall) score at 12 weeks ($p<0.01$). The statistician communicated that this criterion was "not reached" and the study was continued as planned.

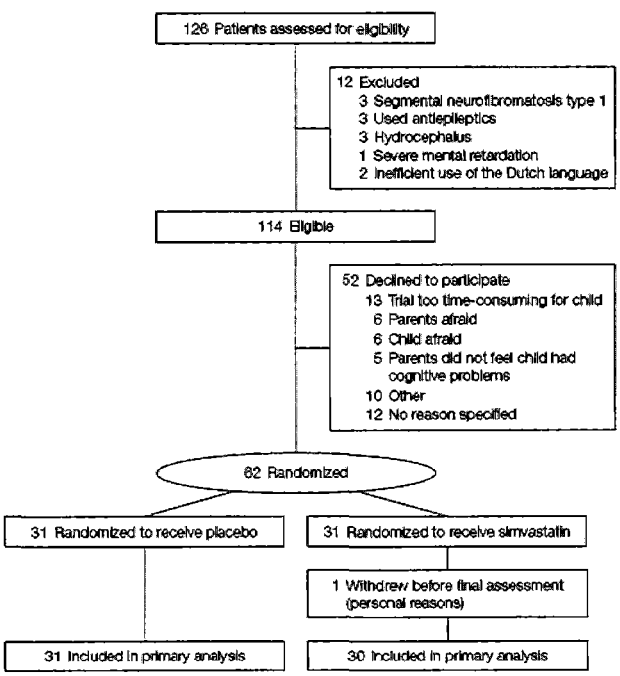

Figure 1: Flow chart of patient inclusion.

Results

Participants

One hundred fourteen children were eligible for this study. Consent to participate was obtained for 62 children (response rate, 54%). The children who participated in the trial (n=62) did not differ significantly from the total eligible group (n=114) on age, sex, frequency of mental retardation, or disease severity (all p>0.3) indicating that they were representative of the total eligible group. The 62 participants were randomly assigned to the simvastatin group (n=31) or the placebo group (n=31) (see Figure 1). The baseline characteristics were similar between the simvastatin- and placebo groups for all baseline parameters except for median age (see tables 1 and 2). Mean (SD) treatment duration was 12 weeks and 3 days (6 days). There were no deviations from random allocation. One participant (2%) in the simvastatin group withdrew from the study after 10 weeks for personal reasons. Three of 62 children (5%), all in the placebo group, were not adherent according to the 80% criterion. We could not retrieve all of the medication jars for 10 of 62 children (16%, 6 in the simvastatin group and 4 in the placebo group).

Table 1. Baseline characteristics of the study groups[a]

	Placebo (n=31) n (%)	Simvastatin (n=31) n (%)
Patient characteristics		
Age at randomization in years, median (IQR) [b]	11.5 (9.4-13.5)	13.2 (11.3-15.2)
Male sex	16 (52)	19 (61)
Full Scale IQ, mean (SD)	85 (15)	88 (15)
NF1 disease severity[c]		
Minimal	10 (32)	12 (39)
Mild	13 (42)	11 (35)
Moderate	8 (26)	7 (23)
Severe	-	1 (3)
Inheritance of NF1		
Familial	14 (45)	12 (39)
Sporadic	16 (52)	19 (61)
Unconfirmed	1 (3)	0 (0)
Socio-economic status[d]		
Low	12 (39)	12 (39)
Middle	9 (29)	9 (29)
High	10 (32)	10 (32)
Total cholesterol, mean (SD), mg/dL	166 (31)	163 (36)
LDL cholesterol, mean (SD), mg/dL	97 (26)	96 (32)
Treatment dose week 9-12[c]		
20 mg/day	NA	12 (39)
40 mg/day	NA	19 (61)
Maximal treatment dose in mg/kg, mean (SD)	NA	0.7 (0.1)

NF1: Neurofibromatosis type 1; LDL: Low-density lipoprotein-Cholesterol; NA: not applicable.
SI conversion factors: to convert cholesterol to mmol/L, multiply by 0.0259
[a]N=62 unless otherwise indicated.
[b]p=0.03 between simvastatin and placebo group
[c]Disease severity of NF1 was scored according to the Riccardi scale modified to exclude cognitive aspects of NF1.4
[d]Socioeconomic status was determined from highest parental occupation or, if not available, highest parental education, and divided into low, middle, or high.

Table 2: Scores on Primary and Secondary Outcome Measures at Baseline and 12 Weeks

	Baseline[a] (mean (SD))	12 weeks[b] (mean (SD))	Univariate difference[b,c] β (95% CI)	Multivariate difference[b,d] β (95% CI)
Primary Outcome Measures				
Rey CFT – delayed recall			0.07 (-0.37 to 0.51)	0.10 (-0.36 to 0.56)
Placebo	-1.6 (0.7)	-1.5 (1.0)		
Simvastatin	-1.7 (0.8)	-1.4 (0.8)		
Cancellation test – speed[e]			-0.27 (-0.74 to 0.20)	-0.19 (-0.67 to 0.29)
Placebo	-0.8 (1.6)	0.4 (1.1)		
Simvastatin	-1.2 (1.8)	-0.1 (1.4)		
Significant Prism adaptation (N (%))[f]			1.57 (0.48 to 5.13)[g]	2.01 (0.55 to 7.37)[g]
Placebo	12 (44)	10 (37)		
Simvastatin	11 (50)	12 (48)		
Average ADC-value ($x10^{-3}$ mm^2/s)[h]			0.01 (-0.12 to 0.14)	0.06 (-0.07 to 0.20)
Placebo	8.03 (0.52)	7.97 (0.50)		
Simvastatin	8.02 (0.44)	7.91 (0.46)		
Secondary Outcome Measures				
Cancellation test – standard deviation[i]			-0.12 (-0.65 to 0.41)	-0.26 (-0.80 to 0.28)
Placebo	-2.7 (1.2)	-1.9 (0.9)		
Simvastatin	-2.8 (1.7)	-2.0 (1.5)		
Stroop – speed[j]			0.34 (-0.36 to 1.04)	0.48 (-0.23 to 1.18)
Placebo	-0.2 (1.8)	0.2 (1.5)		
Simvastatin	-0.5 (2.1)	0.3 (1.9)		
Block design			0.15 (-0.18 to 0.47)	0.10 (-0.24 to 0.44)
Placebo	-1.1 (0.8)	-1.0 (1.0)		
Simvastatin	-0.8 (0.9)	-0.5 (1.0)		
Object Assembly			0.50 (0.05 to 0.95)[k]	0.54 (0.08 to 1.01)[l]
Placebo	-1.1 (1.1)	-0.9 (1.3)		
Simvastatin	-0.8 (1.1)	-0.1 (1.0		
Rey CFT – copy			-0.26 (-0.71 to 0.19)	-0.12 (-0.58 to 0.34)
Placebo	-1.2 (1.2)	-0.7 (1.1)		
Simvastatin	-1.4 (1.3)	-1.0 (1.2)		
Beery VMI			-0.01 (-0.27 to 0.26)	-0.02 (-0.30 to 0.26)
Placebo	-1.2 (0.9)	-1.1 (0.9)		
Simvastatin	-1.2 (0.7)	-1.1 (0.7)		
Judgment of line orientation test			-0.12 (-0.62 to 0.38)	-0.06 (-0.58 to 0.46)
Placebo	-1.6 (1.4)	-1.1 (1.6)		
Simvastatin	-1.1 (1.4)	-0.8 (1.6)		

Abbreviations: NF1: Neurofibromatosis type 1; CFT: Complex Figure Test; Beery VMI: Beery Developmental test of Visual-Motor Integration; ADC: Apparent Diffusion Coefficient.

[a]N=62 (31 placebo, 31 simvastatin) unless otherwise indicated. Values indicate mean (SD) z-score, unless otherwise indicated, in which negative values indicate performance below the normative mean, and positive values performance above the normative mean.

[b]N=61 (31 placebo, 30 simvastatin) unless otherwise indicated; 1 loss to follow up in the simvastatin group before final assessment. Values indicate mean (SD) z-score, unless otherwise indicated, in which negative values indicate performance below the normative mean, and positive values performance above the normative mean.

[c]Values (regression coefficients with 95% confidence intervals) indicate between group differences in scores after 12 weeks, adjusted for baseline scores, obtained from univariate regression analysis.

[d]Values (regression coefficients with 95% confidence intervals) indicate between group differences in scores after 12 weeks, adjusted for baseline scores, age, and sex, obtained from multivariate regression analysis.

[e]Baseline and 12 Weeks: N=29 in the placebo group; only administered if children possessed sufficient rote memory to count groups of up to five dots.

[f]Baseline: N=49 (27 placebo, 22 simvastatin); 7 left-handed children excluded, 6 children excluded due to technical problems including not understanding or adhering to task instructions (N=4). 12 Weeks: N=52 (27 placebo, 25 simvastatin); 6 left-handed children excluded, 3 children excluded due to technical problems including not understanding/adhering to task instructions (N=2).

[g]Odds ratio with 95% confidence interval. N=46 (26 placebo, 20 simvastatin), 6 left-handed children excluded, 9 children excluded due to technical problems including not adhering to task instructions (N=6).

[h]Baseline: N=50 (25 placebo, 25 simvastatin); 2 missing due to artifacts, 10 were not scanned due to limited MRI capacity (random). 12 Weeks: N=46 (23 placebo, 23 simvastatin); 5 missing due to artifacts, 10 were not scanned due to limited MRI capacity (random). A decrease in ADC values indicates lower signal intensity.

[i]Raw score. Baseline and 12 Weeks: N=29 in the placebo group; only administered if children possessed sufficient rote memory to count groups of up to five dots. Larger negative values indicate larger attention fluctuations.

[j]Baseline: N=59 (29 placebo, 30 simvastatin); 12 Weeks: N=58 (29 placebo, 29 simvastatin), only administered if children could read the names of colors. [k]p=0.03. [l]p=0.02.

Effect of simvastatin on outcome parameters

After 12 weeks of treatment, we did not observe a significant difference between the simvastatin and placebo groups on the primary outcome measures (Rey CFT [delayed recall], cancellation test [speed], prism adaptation, and mean brain ADC values). We also did not observe an effect on the secondary outcome measures (cancellation test [standard deviation], Stroop color word test, block design, Rey CFT [copy], Beery developmental test of visual-motor integration and judgment of line orientation), except for a higher score on the object assembly test in the simvastatin group using univariate analysis (adjustment for baseline scores, ß=0.50 [95% CI, 0.05 to 0.95]), as well as multivariate analysis (adjustment for baseline scores, age, and sex, ß=0.54 [95% CI, 0.08 to 1.01]) (table 2).

Paired t-tests revealed that performance after 12 weeks was similar or better than baseline for all tests in both the simvastatin and the placebo groups. In the placebo group, the improvement between baseline and 12 weeks was significant on 4 of 9 neuropsychological outcome measures (cancellation test [speed and standard deviation], Rey CFT [copy], judgment of line orientation), leading to a performance within the normal range on the first 3 tests.

Effect modification

We found that baseline performance on object assembly is a modifier of the effect of simvastatin on this test (p=0.07). Subsequent subgroup analysis showed a significant effect of simvastatin in the group with baseline object assembly test scores ≤ -1SD, (ß =0.80 [95% CI, 0.29 to 1.30]; n=37), but not in the group with baseline object assembly score of >-1 SD (ß =0.47 [95% CI, -0.64 to 1.59]; n=24) indicating that the difference in the object assembly test results between the simvastatin and placebo groups is mostly caused by an increase in score in children with a poor baseline performance in the simvastatin group (figure 2). There was no interaction between improvement on the object assembly task and age.

Safety and effect on cholesterol levels

There were no laboratory adverse events, and no serious adverse events. In total, 5 adverse events were reported by 3 of 31 (10%) children in the simvastatin group: hair loss (1 child after 4, 8 and 12 weeks), muscle weakness (1 child after 8 weeks), and constipation (1 child after 12 weeks) compared with 4 adverse events reported by 3 of 31 (10%) children in the placebo group (dizziness (1 child after 4 and 8 weeks) and constipation (1 child after 8 and 1 child after 12 weeks). None of the reported adverse events reported were judged clinically significant.

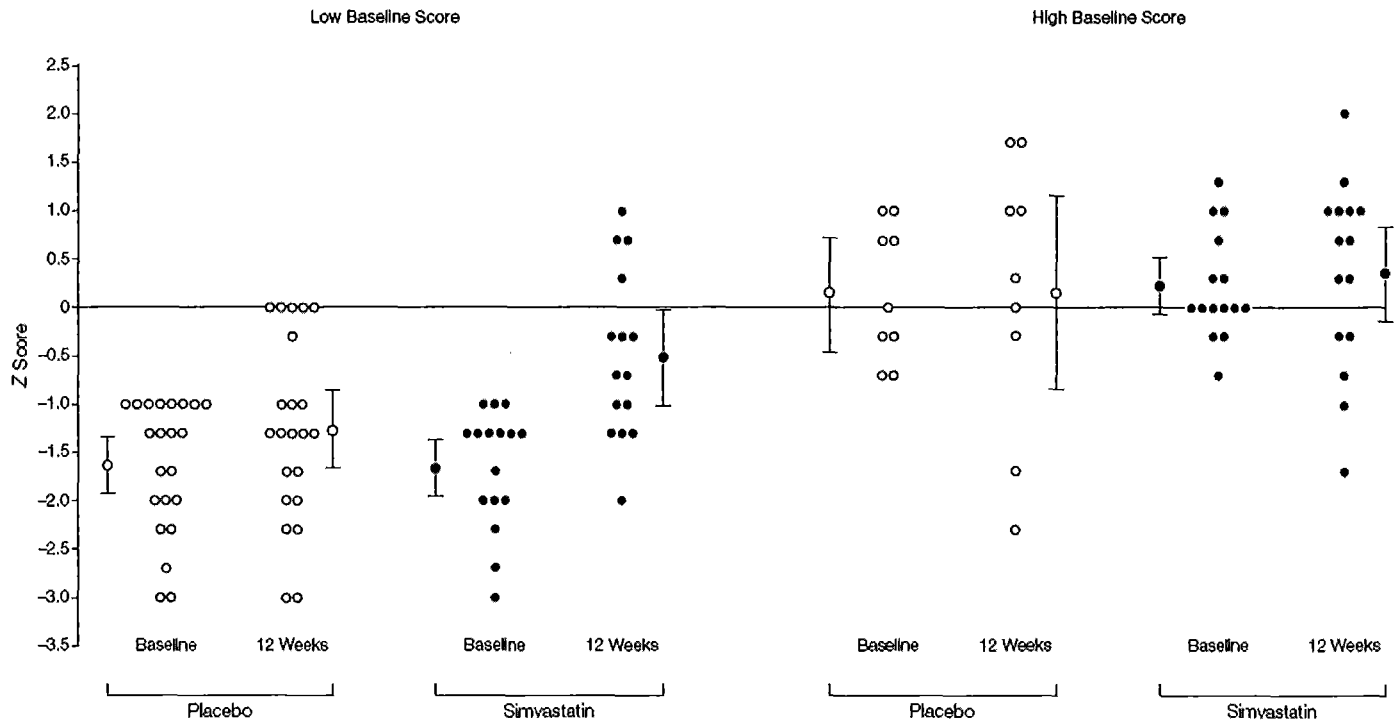

Figure 2: Interaction between baseline score and effect of simvastatin on object assembly test results.

For each subgroup, individual *z* scores and uncorrected group mean *z* scores are provided. For each subgroup, the left range shows scores at baseline and the right range, scores at 12 weeks. For the simvastatin group, n=16 for the low baseline score at baseline but n=15 for the low baseline score at 12 weeks; n=15 for the high baseline score.

For the placebo group, n=22 for the low baseline score, and n=9 for the high baseline score. The difference between the simvastatin and placebo groups after 12 weeks is significant in the groups with low baseline performance (β=0.80; 95% confidence interval, 0.29 to 1.30; p=0.003), but not in the groups with high baseline performance (β=0.47; 95% confidence interval, −0.64 to 1.59).

Error bars represent 95% confidence intervals.

After 12 weeks of simvastatin treatment, total cholesterol levels were reduced by a mean (SD) of 21.1% (10.7%) of baseline values, and low-density lipoprotein cholesterol with 39.4% (15.1%). There was no significant change in high-density lipoprotein cholesterol or triglycerides. The change in low-density lipoprotein cholesterol in the simvastatin group was not significantly related to the dose in mg/kg, sex, or age. The low-density lipoprotein cholesterol level of the children in the simvastatin group who did not return all of their medication jars was decreased by at least 34% (1 not determined because of loss to follow-up).

Comment

We report the results of a randomized, double-blind, placebo-controlled trial to investigate the effect of simvastatin on cognitive functions in children with NF1. We used a carefully selected set of outcomes, including tests resembling measurements shown to be responsive to statins in preclinical studies, tests reflecting the specific neuropsychological deficits in NF1, and objective outcomes such as prism adaptation and brain ADC values, which are insensitive to a placebo or test-retest effect. We did not find an effect of 12 weeks of simvastatin treatment on the primary and secondary outcome parameters, except for higher scores on the object assembly test.

We can conclude post hoc that the power of our study was enough to reject a possible effect on most test. For instance, for the Rey CFT (ß=0.10, se=0.23), we can reject a change larger than 0.56, and for the cancellation test (speed) (ß=-0.19, se=0.24), we can reject a change larger than 0.28. Furthermore, we chose to interpret an improvement of 1 SD as clinically significant, and none of the outcome measures showed a difference between the simvastatin and placebo group of 1 SD or larger. Thus, given the power of the study and the overall negative findings, this study does not provide support for prescribing simvastatin to treat the cognitive deficits of children with NF1.

The object assembly test was the only outcome measure that was significantly improved. Considering that we only found an improvement in object assembly and that we did multiple statistical comparisons without adjusting the p value, this is most likely a spurious finding. It should be noted that the improvement in object assembly was restricted to children who performed poorly at baseline. This specific improvement in the subgroup of children with poor baseline scores is not likely to be related to a practice effect, because children with high baseline scores are expected to benefit most from a practice effect.[25]

The object assembly test measures multiple cognitive domains, but in the context of the entire neuropsychological assessment, along with the clinical behavioral observations made during the assessment, visual synthesis is probably the most damaged cognitive domain. Improved visual synthesis would affect academic performance. For instance, visual synthesis needs to be mastered before children to start reading and spelling, and visual synthesis is an important part of more advanced mathematics.[26,27] However, whether the observed improvement in object assembly is a real effect and whether simvastatin would indeed improve academic achievement remain to be confirmed.

Our study has several limitations. First, the treatment duration used in our study might have been too short to observe a clinically significant recovery in patients with NF1. We based the length of our trial on the observation that statin treatment normalized the plasticity impairment and cognitive phenotype of *Nf1* mice within days,[13] and the observation that treatment of cognitive problems in children can be reached within days to weeks (for instance in the treatment of attention deficits in attention-deficit/hyperactivity disorder, reviewed by Brown et al[28]). However, because precedents for translational trials into cognition are rare, we can not exclude the possibility that the effect of simvastatin on higher cognitive functions in humans would require a longer treatment period than 12 weeks.

Second, the placebo group showed a significant improvement between baseline and 12-week scores on 4 out of 9 neuropsychological outcome measures. This resulted in a performance within the normal range on 3 tests. Because preclinical studies showed that statin treatment did not improve cognitive function in mice that already learned well,[13] it is possible that we reached a performance ceiling, that hampered detection of an effect.

Third, it is conceivable that the therapeutic effect of simvastatin on human brain function was hampered by suboptimal availability due to a first pass effect, or due to inefficient crossing of the blood brain barrier. However, increasing the therapeutic dose does not seem desirable due to the lack of safety studies in children with higher doses, and the increasing risk of side effects observed in adults.[23] Furthermore, the effect of simvastatin on low-density lipoprotein cholesterol at 12 weeks was similar to the decrease achieved after 48 weeks of simvastatin treatment in a previous pediatric study.[16] This indicates that, at least in the liver, the treatment dose was optimal with respect to inhibition of the mevalonate pathway.

Finally, there was a relatively high number of missing data in the neuroradiological and prism adaptation results. Although this reduces the power on these outcome measures there was no indication for a substantial bias because the distribution of observations that were missing did not significantly differ between the simvastatin and placebo groups. For the other outcome measures, the proportion of missing data was negligibly small.

The overall negative outcome of this trial suggests that simvastatin should not be prescribed to ameliorate the cognitive deficits associated with NF1. Further studies to evaluate a longer treatment period and whether the object assembly finding is spurious may be warranted.

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CHAPTER 7.2

Challenges for clinical trials

Author reply

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LETTERS TO JAMA

Challenges for translational trials

To the editor: Krab et al.[1] investigated the possible benefit of statin therapy in children with Neurofibromatosis type 1 (NF1), a genetic disorder which is known to be associated with learning disabilities.[2] Using a randomized, double-blind, placebo-controlled trial it was found that simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, did not improve cognitive function in children with NF1.[1] The motivation for this clinical trial came from the beneficial effect of Lovastatin (another HMG-CoA reductase inhibitor) on cognitive function in the NF1+/- mouse model NF1.[3] These mice are heterozygous for a null mutation in neurofibromin (NF1+/-), and exhibit behavioral disorders that resemble those found in humans, and display deficits in physiological correlates of memory.[3]

First of all, the mechanism for the effect of the statins, such as lovastatin and simvastatin, on cognitive performance in the NF1+/- mouse is not obvious.[4] Statins are cholesterol-lowering drugs, but neurofibromin is not involved in cholesterol metabolism, as it is a tumor suppressor protein. Mutations in neurofibromin are associated with enhanced RAS activity, an oncogene that promotes tumor growth and excessive intracellular signaling in neurons. It has been proposed that statins inhibit the activation of RAS, effectively counterbalancing the enhanced RAS activity associated with neurofibromin.[3] The validity of this proposed mechanism remains to be verified.

Secondly, there are some remarkably differences in the preclinical trial by Li et al, and the clinical trial by Krab et al. Li et al used adult mice, whereas Krab et al. selected children as participants for their trial. Furthermore, a different statin therapy was applied. Lovastatin and simvastatin are HMG-CoA reductase inhibitors with similar, though at some points different, pharmacokinetic profiles.[5] It is also unclear whether the authors considered the effects of nourishments, such as grapefruit juice, on the pharmacokinetics of simvastatin.[5] To what extend are age and pharmacokinetics critical?

The authors correctly conclude that based on the negative outcome of their trial, simvastatin should not be prescribed to ameliorate cognitive deficits associated with NF1. It is difficult to develop a productive and theoretically satisfactory animal model for NF1 because the factors that engender the modeled symptoms or signs of the disorder are often imprecise and incomplete. I wonder what the implications of the study by Krab et al. are for i) the translational value of the current experimental NF1+/- mouse model, and ii) the benefit of statin therapy in general in NF1?

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In Reply: Dr. Jansen raises questions about the rationale of our trial and the value of the *Nf1* mouse model for translational research.[1] It is important to re-emphasize that the NF1 protein is not involved in cholesterol metabolism. NF1 is a negative regulator of RAS activity, and increased RAS signaling has been shown to underlie the learning deficits of *Nf1*$^{+/-}$ mice.[2] The rationale to treat the cognitive deficits with statins was based on 2 fundamental findings in the cancer literature: RAS requires post-translational isoprenylation for proper signaling, and RAS transforming activity can be suppressed by reducing the synthesis of the isoprenyl groups by statins.[1,3,4] The molecular and behavioral deficits of *Nf1*$^{+/-}$ mice can indeed be ameliorated by decreasing RAS activity, either genetically or by administrating farnesyl transferase inhibitors or statins.[1,2]

The pharmacokinetic profile of simvastatin was carefully considered in our study design. Patients and their general practitioners were counseled to avoid the use of medication or food (such as grapefruit juice) that could interfere with the cytochrome-P450-3A4 system in order to avoid significant alterations in simvastatin blood levels.[3] The treatment of central nervous system biochemical deficits has unique considerations compared to treating hypercholesterolemia. For instance, hydrophilic HMG-CoA inhibitors like pravastatin, fluvastatin, and atorvastatin show very limited penetration of the blood-brain barrier.[3] Our rationale to choose the highly lipophilic simvastatin rather than the very similar lovastatin was based on the large amount of safety data available for the use of simvastatin in children.[5] Moreover, simvastatin but not lovastatin is approved for the treatment of familial hypercholesterolemia for children in Europe. Our decision to conduct the trial using children with NF1, rather than adults, was based largely on the well-characterized cognitive deficits of children with NF1. Additionally, early intervention during childhood, the peak period of cognitive development, is likely to maximize the benefits of treatment.

There are 3 major possibilities why the mouse findings could not be replicated in humans: (1) simvastatin is not an adequate treatment of human NF1, (2) simvastatin is an efficacious treatment but the current trial design used a treatment regimen (e.g., daily dose or length of trial) that was below the clinically efficacious threshold, or (3) the *Nf1* mouse model is inadequate. We consider the latter possibility unlikely based on a large literature supporting the translational relevance of the NF1 mouse model. The current data are particularly strong for modeling the cognitive aspects of NF1, as *Nf1*$^{+/-}$ mice show learning and attention deficits in cognitive domains analogous to the patients. The human brain is far more complex than a mouse brain, but it seems likely that the underlying molecular mechanism that is being targeting is conserved across species. It is possible that statin treatment also rescues the learning deficits in *Nf1* flies.[6] Further basic neuroscience and clinical research is therefore needed to investigate how this knowledge can be translated to the patients.

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CHAPTER 8

General Discussion and Future Prospects

General discussion and future prospects

In this thesis, the impact of Neurofibromatosis type 1 (NF1) on daily life was investigated by assessing neuropsychological functioning, school performance, quality of life, and motor behavior in children with NF1. Moreover, NF1-related neuroradiological abnormalities were examined. In addition to characterizing specific NF1-related problems, we tried to identify which aspects of NF1 could be used as outcome measures when investigating potential therapeutic interventions for NF1. In the last and major part of the thesis, several of the outcome measures identified in our studies were incorporated in a randomized, double-blind, placebo-controlled trial to investigate the effect of simvastatin on cognitive functioning in children with NF1 (the NF1 simvastatin trial).

The studies described in this thesis are all carried out in a cohort of NF1 patients aged 7 to 16 years old who are attending the multidisciplinary outpatient clinic of the Erasmus MC – Sophia Children's Hospital Rotterdam, the largest NF1 referral centre in the Netherlands. Most of the data was gathered in the context of the NF1 simvastatin trial, which could potentially create a bias in our data if the patients with the highest burden of cognitive problems would be more inclined to participate than less affected patients. However, non-response analyses indicated this was not the case. Therefore, the results of our studies can offer insight into the general NF1 patient population.

Baseline assessment

School performance

In chapter 3, we revealed that NF1 has a large impact on school performance. An important question outstanding is whether we can predict problems in school performance based on scores in specific domains of the neuropsychological profile of NF1 patients. Ideally, we would want to identify risk factors for school problems at a young age. This would enable us to provide parents and children with a reliable prognosis of school performance, but would also facilitate early intervention in the hopes of preventing school problems later on. Predictive factors can for instance be identified by using regression analysis on data obtained from longitudinal studies, in which children undergo detailed neuropsychological testing at preschool age and quantitative assessment of school performance at an older age. However, such a longitudinal study requires a long follow-up period. As a short-term alternative, we can use the

cross-sectional data from chapter 3 to explore the relationship between neuropsychological functioning and school performance.

The results of this preliminary analysis are displayed in supplementary table 1 at the end of the discussion. The high correlations between learning efficacies and IQ can be explained by the fact that more than half of the learning disabilities in NF1 occur in the context of a lowered IQ. In addition, we observed a link between performance on some, but not all, of the neuropsychological tests frequently affected in NF1 and school scores, which indicates these neuropsychological tests might be relevant outcome measures that can be used in studies investigating the effect of potential treatments for cognitive problems in NF1. The lack of a clear association between attention deficits and school performance is unexpected, as a relationship between ADHD and literacy based learning disabilities is suggested in NF1 patients as well as in the general population.[1, 2] Due to the limited sample size available for these analyses, the correlation of a *combination* of individual factors with school scores can only be estimated from data of larger studies.

After identifying predictive factors for school performance in longitudinal studies, an important follow up question is whether school problems can actually be reduced, and if so, to what extent, by targeting deficits in these predictive domains with either (tailored) remedial teaching programs or drug therapies. The ideal method to study these effects is by performing a randomized trial. However, as remedial teaching is often already implemented in regular care, it is not expected that parents will participate in a randomized trial which incorporates a control group without remedial teaching. Therefore, one would have to refer to before- and after measurements.

Health Related Quality of Life

In chapter 4, we observed that NF1 children, in contrast to their parents, report difficulties in only one domain of Quality of Life of the Child Health Questionnaire (CHQ). Although a discrepancy between ratings of parents and children does not imply one of them is more accurate,[3] these results are intriguing. Only one other group has investigated child self-reports in NF1 so far, and this study showed children do report significant problems on multiple domains of the TNO-AZL Quality of Life questionnaire (TACQOL).[4] The discrepancy between our study and that of Graf et al. could be caused by (cultural) differences between patient populations, but also by methodological distinctions between the questionnaires used. The CHQ assesses the prevalence of problems, whereas scores on the TACQOL are determined by

the amount of distress related to these problems regardless of the prevalence. Possibly, children with NF1 cannot accurately perceive or report either one of these aspects, which might be related to the 'positive illusory bias' mentioned in the introduction.[5-7] A way to investigate whether methodological differences can explain the differences in problem scores across questionnaires, and to investigate whether this sensitivity to the way questions are posed is NF1-specific, would be to administer both the CHQ and the TACQOL questionnaires to both our study population and to a group of healthy control children and compare scores.

We revealed that parent-reported Health Related Quality of Life scores are strongly sensitive to behavioral problems (rated by teachers). This insight suggests an opportunity to influence not only behavior itself but also quality of life in general by addressing these behavioral problems, for instance with specific medication, behavioral training programs or family education. A problem with this approach is that the children with NF1 themselves did not seem to notice behavioral problems, as they scored their own behavior as significantly better than average. This discrepancy could again be explained by the positive illusory bias. It is not clear whether standard behavioral training programs would be beneficial for the self-perception of children with NF1. One can imagine that confronting children with a positive illusory bias with their inadequate behavior could even have a negative rather than positive impact on their self-esteem or quality of life, which calls for a careful approach.

From our study on school performance we can conclude that school problems pose a major burden for parents and children, as children frequently need multiple types of remedial teaching and often have to repeat a grade. However, an unexpected finding of our study on Quality of Life was that these school problems do not seem to contribute to Health Related Quality of Life scores. It could be that our school scale was not sensitive enough to pick up a relationship between school problems and Health Related Quality of Life scores. Another possible explanation is that the parent's percept of their child's school performance is not linearly related to their child's objective academic achievement. Parents of a child that is thriving in a special education class could experience less school-related concerns than parents of a child struggling to keep up with regular education. It would be interesting to incorporate parental opinions of their child's school performance as a covariate in future studies.

Motor functioning

In **chapter 5**, we showed that children with NF1 display deficits on fine motor functioning and visual integration, and adaptation of hand movements to prism glass distortion. Our study did

not reveal a specific anatomical correlate of these motor problems in NF1. By making use of mouse models for NF1, future studies could assess the functional integrity of brain areas involved in motor behavior. In order to determine whether cerebellar function is affected in NF1 we could assess cerebellar synaptic plasticity, for instance in the GABA-agric Purkinje cell / deep cerebellar nuclei synapses. Also, *Nf1* mice could be tested with cerebellum-specific motor adaptation tasks, such as conditioning of eyeblink responses.[8] The benefit of eyeblink conditioning is that it can be assessed in mice as well as patients.[9, 10] If *Nf1* mice and humans turn out to have parallel deficits in eyeblink conditioning, this test can serve as a unique translational outcome parameter in future studies assessing the effect of potential drug therapies on motor functioning in *Nf1* mice and NF1 patients.

Unidentified Bright Objects

Our study on Magnetic Resonance (MR) abnormalities in NF1 has gained insight into the nature of T2-weighed hyperintensities **(chapter 6)**. However, the effect of these Unidentified Brain Objects (UBOs) on brain functioning and cognition is still unclear. Previous studies investigating the relationship between UBOs and cognition mostly identified UBOs visually on conventional T2-weighed MR images.[11-19] However, as we and other studies showed that Apparent Diffusion Coefficients (ADC values) are also elevated in normal-appearing brain areas in NF1 patients,[20-23] visual identification might not be an accurate parameter for brain pathology in NF1. The information gathered in our studies allows for a more detailed investigation of the relationship between NF1-specific brain pathology and cognition.

Preliminary analysis of our data is shown in supplementary table 1. Although there is little correlation between average ADC-value and neuropsychological performance, there does seem to be a tendency for a *positive* relationship between average ADC value and learning efficacies for technical reading, comprehensive reading and spelling. Although these results should be viewed in the light of the small study group and the large amount of statistical comparisons made, it must be noted that only one out of the 16 comparisons made for the neuropsychological tests has a negative correlation coefficient. If anything, our data suggest a positive relationship between ADC values and cognitive functioning, with higher ADC values (which are indicative of UBOs) related to higher test scores. This is opposite to all previous reports in literature, and there is no straightforward explanation for this. One would imagine that myelin disturbances impair axonal conductance, and lead to inadequate neuronal signaling and impaired cognitive functioning. It is tempting to speculate that UBOs might be indicators of a compensatory mechanism to reduce increased neuronal inhibition. NF1 heterozygous knockout mice do not

seem to display UBOs,[24] which makes it difficult to assess the effect of UBOs on neuronal functioning.

Potential outcome measures: prism adaptation and ADC values

Part of the reason we conducted the neurophysiological and neuroradiolocical studies described in chapter 5 and 6 was to identify possible objective, placebo-insensitive outcome measures to assess the effect of potential therapies on cognitive functioning in NF1 patients.

Performance on the prism adaptation test could potentially be more sensitive to changes in neuronal functioning than neuropsychological tests for higher order cognitive skills, because the task depends upon a limited neuronal circuit,[25] and deficits can not easily be compensated for.[26, 27] Although some children were excluded because they did not understand or adhere to task instructions or were left-handed, overall the prism adaptation test is easy to perform,[28] rapid, and can be quantified objectively. As adaptation of hand movements to prism glass distortion was found to be impaired in NF1 patients, we decided to incorporate this test as an objective outcome measure in our clinical trial.

As described in chapter 6, ADC values were significantly higher in children with NF1 than in controls. In addition, we showed that the measurement of ADC values was reproducible, and NF1 children were able to undergo MR investigation without needing sedation. Previous studies indicate that UBOs can resolve over time, and that ADC values are more sensitive to brain pathology in NF1 than UBOs.[21, 29, 30] Thus, it was conceivable that if statins reduce brain pathology in NF1, this could be picked up by measuring ADC values. Therefore, we incorporated ADC values as an objective outcome measure in our trial.

Investigating the effect of statins on cognition in children with NF1

In chapter 2, we reviewed the insights in the molecular and cellular mechanisms underlying the cognitive deficits in NF1 and affiliated diseases among the neuro-cardio-facial-cutaneous and Hamartoma syndromes. Research in mouse mutants has revealed that the cognitive deficits of these diseases evolve around elevated activity of the RAS/ERK and RAS/PI3K/MTOR pathways, which leads to changes in synaptic plasticity. RAS activity can be decreased by attacking it's Achilles' heel: its requirement to be isoprenylated.[31] Statins can decrease the

production of isoprenyl groups by inhibiting HMG-CoA reductase, the rate-limiting enzyme in the mevalonate synthesis pathway. Statins are cholesterol-lowering drugs, used by millions of people worldwide, and show very good safety profiles in adults and children.[32, 33] A breakthrough in the pursuit of a treatment for the cognitive deficits in NF1 patients was made when it was discovered that short-term lovastatin treatment could reduce RAS activity, and rescue the deficits in synaptic plasticity, learning, memory and attention in *Nf1* mice.[34] The favorable safety profile of statins offers a unique opportunity to translate these preclinical findings and to assess the effect of a targeted treatment on cognitive function in NF1 patients.

In chapter 7, we report the results of the first randomized, placebo-controlled, double blind trial to assess he effect of statins on cognitive function in children with NF1. Sixty-two children with NF1 aged 8 to 16 years were treated with simvastatin or placebo once a day for 12 weeks. The effect of simvastatin was assessed using neuropsychological, neurophysiological and neuroradiological outcome parameters, carefully selected from the tests found to be impaired in our NF1 patient population in the baseline studies performed in chapters 3, 5 and 6.

We did not observe an effect of simvastatin on the primary outcome measures (Rey Complex Figure test, Cancellation test [speed], Prism Adaptation and average brain ADC-value). On the secondary outcome measures, we found a significant improvement in the simvastatin group in object assembly scores (ß=0.54, CI: 0.08–1.01), which was specifically observed in children with poor baseline performance (ß=0.80, CI: 0.29–1.30).

The changes in object assembly found in our study could be a spurious finding, and need to be verified in other studies. An important question is what are the consequences of improvement in object assembly for daily life. Object assembly is postulated to assess visual analysis (the ability to synthesize an image from fragmented visual information),[35] which is an important prerequisite ability for reading and spelling, and is used in advanced mathematical problem solving.[36, 37] Unfortunately, preliminary analysis of our data on school performance does not reveal any correlation between performance on object assembly and learning efficacies on technical reading, comprehensive reading, spelling or mathematics in children with NF1 (all R 0.0-0.1). Thus, we cannot say whether improvements in object assembly would be beneficial to children with NF1 on the long run. Another concern is that our trial included outcome measures that tap into some of the other skills required for performance on the object assembly task, such as visual synthesis (Block Design test) and visual motor coordination (Beery VMI test), but these were not changed in our trial.

The overall conclusion of our trial was that 12-week simvastatin treatment does not improve cognitive functioning in NF1 patients.

Factors confounding trial outcome

Several factors could have attributed to the negative outcome of the NF1 simvastatin trial.

First, the treatment duration used in our study might have been too short. We based the length of our trial on the observation that statin treatment normalized the plasticity impairment and cognitive phenotype of *Nf1* mice within days,[34] and the reports that clinically significant reduction of cognitive problems in children can be reached within days to weeks (for instance in the treatment of attention deficits in ADHD, reviewed by Brown et al [38]). However, since precedents for translational trials into cognition are rare, we cannot exclude the possibility that the effect of simvastatin on higher cognitive functions in humans would require a longer treatment period than 12 weeks.

Second, relatively little is known about the comparison of pharmacokinetic and pharmacodynamic findings in mice and humans. It is conceivable that the therapeutic effect of simvastatin on human brain function was hampered by suboptimal availability of the drug or due to inefficient crossing of the blood brain barrier.

With respect to the availability of the drug, it is known that statins undergo a large first pass effect.[39] Statins are generally administered orally, as an inactive lactone prodrug that is converted into an active hydroxyacid form by carboxylesterases. This conversion is much more rapid in rodents than in humans,[40, 41] which potentially results in differences in the availability of the drug. In humans, the inhibition of HMG-CoA reductase in plasma reaches a peak after a few hours, and within approximately 8 hours fall back to baseline levels.[39] It is unclear if this relatively short time interval is sufficient to reach a sustained effect on protein isoprenylation. In our trial, we administered simvastatin in the morning in order to time the peak of statin activity during school hours.

The way statins penetrate the blood brain barrier is still unclear, and does not solely depend upon the lipophilicity of the drug. The lactone and acid forms are shown to interact differently with efflux and uptake transporters present in the blood brain barrier.[42] The brain penetration of the lactone form, but not the active acid form, is limited by the drug transporter P-glycoprotein.

Despite this, only a limited amount of the active acid form seems to penetrate the blood brain barrier.[43] Brain carboxylesterases can convert the lactone form to the acid form, but conversion does not seem to take place the other way around. It is unclear which form of statins is the most important inhibitor of brain HMG-CoA reductase. Part of the studies in the paper of Li et al. were performed using subcutaneous injections of the acid form (the RAS activity assays, the plasticity experiments and the task for learning and memory).[34] Differences in the way statins are administered might influence the effect on the mevalonate pathway in the brain.

Regardless of the way statins reach the brain, statin treatment has been shown to reduce brain cholesterol synthesis in mice,[44] and thus statins potentially affects isoprenyl concentrations in the neurons. However, their influence on brain cholesterol turnover in hyperlipidemic patients is unclear.[45, 46] The effect of simvastatin on brain cholesterol synthesis was not assessed in our study but could be determined by measuring 24(S)-hydroxycholesterol, a serum marker for brain cholesterol metabolism that is suggested to be responsive to simvastatin treatment.[45, 47]

Third, we observed large improvements on the neuropsychological outcome measures in both treatment groups, which might be attributable to a placebo-effect,[48] but also to practice effects resulting from repeated assessment with the same tests. Practice effects can be observed even when applying parallel versions of a test.[49] Although the placebo-controlled design of our trial should account for these effects, the mean score on 3 out of 9 neuropsychological outcome measures was increased up to a normal score in the placebo group after 12 weeks. Children in the simvastatin group would have had to improve beyond the normative average before a treatment effect could have been identified. As statins do not seem to improve cognitive function in NF1 and wild type mice beyond normal,[34] it is possible that a performance ceiling was reached for these measures, which might have hampered identification of additional effects of simvastatin.

Finally, it cannot be excluded that the outcome measures used in our study were too specific to pick up overall improvements in daily life functioning. Potentially, subtle changes in multiple cognitive domains could together result in substantial changes in daily life functioning and subjective well-being without significantly improved scores on tests for the separate cognitive skills.

Recommendations for future trials

Despite the fact that our NF1 simvastatin trial did not provide evidence that short-term simvastatin treatment has an effect on cognitive functioning in NF1 patients, the favorable safety profile of statins, and the limitations of this first trial, do call for further randomized double-blind placebo-controlled trials. Below, we discuss some important recommendations for the design of these future studies.

Treatment period

The major recommendation for follow-up studies is to treat children for a much longer period. A longer treatment duration in future trials may attenuate the placebo effect, wear off practice effects, enable a longer exposure to the highest therapeutic dose, and allow for a longer time for the brain to restore function. Moreover, a longer treatment duration would allow inclusion of real-life outcome measures such as school performance, behavior and quality of life. A follow-up trial with a treatment period of 1 year is currently in preparation at the Erasmus MC – Sophia Children's Hospital Rotterdam.

Treatment dose

Increasing the therapeutic dose does not seem desirable due to the lack of safety studies in children with higher doses, and the increasing risk of side effects observed in adults.[50] In addition, the effect of simvastatin on low-density lipoprotein cholesterol at 12 weeks was similar to the decrease achieved after 48 weeks of simvastatin treatment in a previous pediatric study.[32] This indicates that, at least in the liver, the treatment dose was optimal with respect to inhibition of the mevalonate pathway. Moreover, we did not observe any relationship between the dose of simvastatin and decrease of low-density lipoprotein cholesterol (chapter 7), or the change in object assembly score (unpublished observations).

Patient selection

It might be recommended to select individual patients based on baseline impairments, because our subgroup analysis suggests that patients with low baseline scores might respond better to simvastatin therapy. Excluding children with normal scores would also decrease the change of children reaching a ceiling in their performance. However, drawbacks of patient selection are that it introduces a selection bias, which lowers the generalisability of the study, and limits the number of eligible patients, especially when selecting for performance deficits in multiple outcome measures.

Although the diagnosis of NF1 is still based only on clinical criteria, it does seem important to include patients only if the NF1 diagnosis has been confirmed with genetic testing. Despite the fact that patients with affiliated disorders in the RAS pathway might also respond to statin treatment, the magnitude of response might differ according to the specific gene affected, which would add to the variability in the study. The fact that even with thorough clinical assessment, patients with other disorders can erroneously receive an NF1 diagnosis is illustrated by the unexpected finding of a PTPN11 T468M point mutation, indicative of Noonan Syndrome or LEOPARD syndrome,[51, 52] in one of the participants of the NF1 simvastatin trial.

Selection of outcome measures

In general, it is recommended to select a limited amount of primary outcome measures, in order to prevent having to correct statistical findings. Outcome measures used in future studies need to have a good test-retest reliability, as this is a major determinant of the power of the study. It is of pivotal importance to select tests on which NF1 patients show impairments (although the exact cut-off for the level of impairment can be adjusted), in order to have room for improvement and to prevent ceiling effects.

Another important recommendation is to select outcome measures that have a high predictive validity, so that changes on these tests can be interpreted in terms of their consequences for daily life functioning. Our preliminary analysis indicates that some neuropsychological tests are more suitable in this respect than others. For instance, it might be recommended to include total IQ and neuropsychological tests for language, but to drop the Judgment of Line Orientation Test as it does not seem to correlate to performance in any academic domain. Although object assembly does not seem to predict school performance in our population, we do recommend incorporating it as an outcome measure in future trials, in order to examine whether our current results can be replicated, and to be able to compare results across studies.

As indicated above, a longer treatment duration would enable the assessment of the effect of statins on daily life functioning. Considering the problems identified in our NF1 patient population in our studies on school performance and quality of life, we recommend to include detailed quantitative assessment of academic achievement, as well as standardized validated questionnaires on behavior, quality of life, and also self esteem, from the perspective of parents, teachers and children.

Behavior was assessed in our trial, but was not selected as an outcome measure because scores at baseline were less than 1 SD below normative values. Our results indicate that self-reported behavior might be sensitive to simvastatin treatment, as children's scores on a validated behavioral questionnaire (the Youth Self Report Form, Achenbach) were marginally significantly more improved in the simvastatin group ($p=0.06$ on multivariate analysis). Parent and teacher's ratings revealed no significant differences between the simvastatin and placebo groups, although in both groups the scores improved substantially. Because the number of children that were old enough to fill out this questionnaire (>11 years) and returned their forms at baseline as well as after 12 weeks was small, these results should be interpreted with care, but do stress the importance of including behavioral questionnaires in future studies.

Based on progressive insight gathered in our trial, we do not recommend incorporating the prism adaptation task in future trials. On repeated assessment, the missing data on this test were relatively large. Also, when closely examining the prism adaptation data in the placebo group, it seems that performance on this task is not as stable as we had anticipated. On the group level, the distribution of children over the 'adapting' and 'not-adapting' categories was similar at baseline and after 12 weeks. However, on an individual level, performance is very variable, with only 13 out of 26 children falling into the same category at both time points.

Although 12 weeks simvastatin therapy did not have a significant effect on mean brain ADC values, this could be confounded by the short treatment duration. However, the potential benefits of incorporating the objective investigation of brain pathology in future trials should be balanced against high costs and time-consuming nature of this measurement, and its low predictive validity for school problems and neuropsychological problems.

Future trials are strongly recommended to incorporate determination of 24(S)-hydroxycholesterol levels in serum to assess the effect of simvastatin on brain cholesterol synthesis.

Other applications of statins

Applications of statins for other NF1-related problems

Recent studies reveal other potential therapeutic options for statins in NF1 besides treatment of cognitive deficits. Oral lovastatin treatment can rescue the delayed bone repair of mice with

conditional bi-allelic inactivation of neurofibromin in the developing limbs and cranium, probably via repression of ERK-activity.[53] In addition, in NF1 Malignant Peripheral Nerve Sheet Tumor cell lines, lovastatin and a farnesyl transferase inhibitor were found to have a synergistic effect, and together reduced RAS isoprenylation, decreased cell proliferation and induced apoptosis. Single administration of either agent did not have this effect.[54] It would be very interesting to assess the effect of statins on bone repair and Malignant Peripheral Nerve Sheet Tumors in clinical trials. However, as these complications of NF1 is rare, they need to be investigated in separate studies.

Applications of statins for cognitive impairments in other diseases

As reviewed in chapter 2, statins do not only offer prospects of developing a treatment for NF1. Statins are also of great interest for other diseases in the RAS pathway such as the Neuro-Cardio-Facial-Cutaneous syndromes. In addition, statins could potentially target the molecular disturbances underlying the Hamartoma syndromes, which are not only co-regulated by RAS, but also critically dependent upon RHEB, another member of the RAS family that requires isoprenylation. However, awaiting further clinical trials, a first step should be to obtain a proof of principle in the preclinical models of these diseases.

The potential effect of statins on for instance Alzheimer's disease, Multiple Sclerosis, and the incidence of stroke is of great interest to the general population.[35] It is also tempting to speculate whether statins could improve cognitive functioning in otherwise healthy humans. This seems unlikely, as statins do not alter the learning phenotype of wild type mice or flies.[34, 55] However, in another study, in acute application of statins to brain slices of wild-type mice did not seem to affect long term potentiation.[56] The reports on the effect of statins on cognition in humans are conflicting as well. Although several case reports indicate statins have a negative effect on cognition,[57] results from clinical trials in patients with hypercholesterolemia and mild cognitive decline are controversial. Some of these trial indicate that statins lead to relatively lower learning capacities,[58, 59] but others do not find an effect, [60, 61] or report a favorable effect on cognitive decline.[62] In all, it seems that the effect of statins on the brain will remain a hot topic for quite some time.

Conclusion

Our studies indicate that NF1 has a large impact on daily life functioning, as NF1 children show substantial problems in school performance and motor functioning and, at least according to parents, have a lower quality of life. Awareness of the problems associated with NF1 may facilitate timely recognition and appropriate intervention. Our studies have pointed out several potential targets for structural support, such as behavioral problems. Also, we have identified several outcome measures that can be used to assess potential treatments for cognitive deficits in NF1. Although the preclinical studies were promising, short-term statin treatment did not improve cognitive functioning. Still, further clinical trials are needed to reveal whether long-term statin treatment can improve school performance, behavioral problems, and quality of life in children with NF1.

Supplementary table 1. Correlations between Learning efficacies and performance on Neuropsychological tests and average ADC-value of the brain.[a,b]

	Didactic domain[c]			
	Technical Reading	Comprehensive Reading	Spelling	Mathematics
Neuropsychological domain				
IQ	0.5	0.8	0.5	0.7
Memory				
Verbal short term[b]	-	-	-	-
Verbal long term	-	-	-	-
Non-verbal	-	-	-	-
Language				
Expressive	0.3	0.5	0.3	0.3
Receptive	0.4	0.6	0.5	0.5
Visual spatial skills				
Visual integration	0.4	0.5	0.5	0.4
Visual motor integration	0.4	0.5	0.4	0.6
JLO	-	-	-	-
Executive skills				
Rote memory	-	-	-	-
Divided attention	-	-	-	-
Verbal fluency	0.3	0.4	0.4	0.4
Concept formation	0.3	0.4	-	0.4
Preservations	-	-	-	0.3
Attention				
Sustained	-	-	-	-
Selective	-	0.3	-	-
Neuroradiological parameter				
Average ADC-value	0.3	0.3	0.4	-

[a]Values represent Pearson's R. Only significant correlations ($p<0.05$) are displayed; '–' indicates no significant correlation.

[b]The average ADC-value did not correlate significantly with any neuropsychological test except for verbal short term memory (R=0.3).

[c]N = 39 to 51, depending upon the availability of the didactic information and neuroradiological assessment.

JLO: Judgment of Line Orientation Test; ADC: Apparent Diffusion Coefficient.

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1. **Lianne C. Krab**, Arja De Goede-Bolder, Femke K. Aarsen, Saskia M.F. Pluijm, Marlies J. Bouman, Jos N. van der Geest, Maarten Lequin, Coriene E. Catsman-Berrevoets, Willem Frans M. Arts, Steven A. Kushner, Alcino J. Silva, Chris I. De Zeeuw, Henriëtte A. Moll, Ype Elgersma. The effect of simvastatin on cognitive functioning in children with Neurofibromatosis type 1: a randomized, double-blind, placebo-controlled trial. *13th European Neurofibromatosis Meeting, Killarney, Ireland, 2008 (oral presentation).*

2. **Lianne C. Krab**, R. Oostenbrink, Arja de Goede-Bolder, Femke K. Aarsen, Ype Elgersma, Henriëtte A. Moll. Health Related Quality of Life in children with Neurofibromatosis Type 1 - Contribution of demographic factors, disease related factors, and behavior. *13th European Neurofibromatosis Meeting, Killarney, Ireland, 2008 (oral presentation).*

3. **L.C. Krab**, F.K. Aarsen, A. de Goede-Bolder, C.E. Catsman Berrevoets, W.F. Arts, H.A. Moll, Y. Elgersma. Impact of Neurofibromatosis type 1 on school performance. *2007 Neurofibromatosis Conference: Models, mechanisms and therapeutic targets, Park City, Utah, USA, 2007 (poster presentation)*

4. **L.C. Krab**, F.K. Aarsen, A. de Goede-Bolder, C. Catsman-Berrevoets, H.A. Moll, Y. Elgersma. Impact of Neurofibromatosis type 1 (NF1) on school performance. *12th European Neurofibromatosis Meeting, Lisbon, Portugal, 2007 (oral presentation).*

5. **Lianne C. Krab**, MSc[1,2], Rianne Oostenbrink, MD PhD[2], Femke K. Aarsen, MA[3], Arja de Goede-Bolder, Coriene E. Catsman MD PhD[3], Henriëtte A. Moll, MD PhD[2], Ype Elgersma, PhD. Behavior and Health related Quality of Life (HRQoL) in school-aged children with Neurofibromatosis type 1 (NF1). *12th European Neurofibromatosis Meeting, Lisbon, Portugal, 2007 (poster presentation).*

6. **L.C. Krab**, F.K. Aarsen, Arja De Goede-Bolder, C. E. Catsman-Berrevoets, M. Lequin, W.F.M. Arts, H.A. Moll, Y. Elgersma. Resultaten van medicamenteuze interventie van leerstoornissen bij kinderen met NF1 in een dubbelblinde placebo gecontroleerde trial. In: symposium Neurofibromatose type 1: van muis naar mens. *29e Congres Kindergeneeskunde, Veldhoven, Nederland, 2007 (oral presentation).*

7. **L.C. Krab**, A. De Goede-Bolder, C. Catsman-Berrevoets, F.K. Aarsen, H.A. Moll, Y. Elgersma. Impact van NeurofibromatoseType 1 (NF1) op de schoolcarrière. *28e Congres Kindergeneeskunde, Veldhoven, Nederland, 2006 (poster presentation).*

List of Publications

ARTICLES IN THIS THESIS

1. **Lianne C. Krab***, Susanna M.I. Goorden*, Ype Elgersma. Oncogenes on my mind: ERK and MTOR signaling in cognitive diseases. *Trends in Genetics, 2008;24(10): 498-510*
   *Authors contributed equally.

2. **Lianne C. Krab**, Femke K. Aarsen, Arja de Goede-Bolder, Coriene E. Catsman-Berrevoets, Willem F. Arts, Henriëtte A. Moll, Ype Elgersma. Impact of NF1 on school performance. *Journal of Child Neurology, 2008;23(9): In Press (doi: 10.1177/0883073808316366)*

3. **Lianne C. Krab**, R. Oostenbrink, Arja de Goede-Bolder, Femke K. Aarsen, Ype Elgersma, Henriëtte A. Moll. Health Related Quality of Life in children with Neurofibromatosis Type 1: Contribution of demographic factors, disease related factors, and behavior. *The Journal of Pediatrics, 2008; In Press (doi: 10.1016/j.jpeds.2008.08.045)*

4. **Lianne C. Krab**, Arja de Goede-Bolder, Femke K. Aarsen, Henriëtte A. Moll, Chris I. De Zeeuw, Ype Elgersma, Josef N. van der Geest. Motor learning in children with Neurofibromatosis type I. *Submitted (2008)*

5. S.J.P.M. van Engelen, **L.C. Krab**, H.A. Moll, A. de Goede-Bolder, S.M.F. Pluijm, C.E. Catsman-Berrevoets, Y. Elgersma, M.H. Lequin. Quantitative differentiation between healthy and disordered brain matter in Neurofibromatosis type I patients using Diffusion Tensor Imaging. *AJNR American Journal of Neuroradiology 2008;29(4): 816-22*

6. **Lianne C. Krab**, Arja De Goede-Bolder, Femke K. Aarsen, Saskia M.F. Pluijm, Marlies J. Bouman, Jos N. van der Geest, Maarten Lequin, Coriene E. Catsman-Berrevoets, Willem Frans M. Arts, Steven A. Kushner, Alcino J. Silva, Chris I. De Zeeuw, Henriëtte A. Moll, Ype Elgersma. The effect of simvastatin on cognitive functioning in children with Neurofibromatosis type 1: a randomized, double-blind, placebo-controlled trial. *JAMA, 2008;300(3): 287-294*

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About the author

Lianne Caroline Krab was born in Velsen on July 31, 1980, and grew up in Assendelft and Uitgeest. She obtained her VWO-degree at the Bertrand Russell College in Krommenie in 1998, after which she attended University College Utrecht, the international Honors College of Utrecht University, for two years, supported by the 'Fundatie van de Vrijvrouwe van Renswoude te Utrecht'.

In 2000 Lianne started medical school at the Erasmus University Rotterdam, and in 2001 she was admitted to the parallel Master of Science in Neuroscience trajectory. In her traineeship in the lab of dr. Y. Elgersma she worked on the projects 'Identification of genes causing learning disabilities in children with NF1' and 'Relationship between motor learning and cognitive functioning in children with NF1' (in collaboration with general pediatrician drs. A. de Goede-Bolder and neuroscientist dr. J.N. van der Geest, respectively). In 2004 she obtained her doctoral degree in medicine, and in 2005 her Master of Science in Neuroscience degree.

In April 2005 Lianne began her PhD research on the NF1 simvastatin project, in collaboration with the multidisciplinary CoRe (Cognitive Research) Team of the Erasmus MC – Sophia Children's Hospital Rotterdam. During her PhD trajectory, she was co-applicant on several grants for NF1 research, and received the Jan C. Molenaar Award for the oral defense of her grant-proposal for the scientific committee of the Sophia Kinderziekenhuis Fonds in 2005. Lianne presented her work at national conferences and symposia, and at the international NF conferences in Portugal (with personal funding of the Neurofibromatose Vereniging Nederland), Utah (USA), and Ireland. In addition, she was an invited speaker at meetings of NF patient lay groups and NF1 clinical research teams in the Netherlands, Norway, Belgium, Germany and Ireland. She wrote several contributions for the patient magazine of the Neurofibromatose Vereniging Nederland.

In May 2008 Lianne started her medical internships at the Erasmus University Rotterdam. In 2010 she will obtain her Medical Degree, after which she plans to remain involved as a clinician and scientist in the search for therapies for children with genetic disorders affecting cognition. She lives in Leiden with her partner Arjen in their 1930s house which they rebuilt together.

Het maken van dit proefschrift was flink aanpoten, waardoor mijn vrienden en familie mij de afgelopen jaren heel wat uurtjes hebben moeten missen. Speciale dank aan Ophirah, voor een spoedcursus eigen prioriteiten stellen... en nog veel meer. Pap, mam, hoewel jullie bang waren dat ik door al dat onderzoek zou vergeten dokter te worden, zijn jullie me altijd blijven steunen. Aan jullie heb ik mijn voelsprieten te danken, die zich heerlijk op hun plek voelen in de kliniek! Mariska, Maurits, Fiona, we leren elkaar steeds beter kennen. Ik ben trots als zusje in onze groeiende familie te staan. Maaike, schoonzusje, bedank voor het nalezen van mijn stukken en de gezellige klets (vooral op zondag, heb je 't in de gaten?).

Arjen, mijn maatje, tijdens alle (promotie)stress bleef jij als een rots in de branding, rustig, oprecht en toegewijd. Wat bof ik dat ik jou nu al gevonden heb!

Zo, en nu eerst een ontzettend goede dokter worden!

Lianne

hebben we geworsteld om de MRI's bij het onderzoek te krijgen. Het is gelukt! Bedankt voor alle uitleg op mijn (zeer waarschijnlijk heel vaak dezelfde) basale radiologische vragen. Nanda, bedankt voor al je administratieve ondersteuning.

Het Elgersma lab, jullie zijn inmiddels met zovelen dat ik het amper bij kan houden. Voor alle leden: bedankt dat ik de pure basale wetenschap in al haar schitterende maar vaak ook harde facetten van zo dichtbij heb mogen ervaren. Nils, ik was met veel plezier je kamergenote in ons mini-lab met hersenplakjes achter mijn stoel. Met jou kun je altijd en over alles diepgaand discussiëren. Ook manlijk advies over vrouwlijke problemen ging je niet altijd uit de weg. Wat was het stil hé, nadat ik weg ging ☺. Geeske, we hebben elkaar heel wat af moeten tasten. Enorm veel respect voor je doorzettingsvermogen en het plezier waarmee je je zo ontzettend veel labtechnieken eigen hebt weten te maken en waarmee je je kennis aan anderen doorgeeft. Petra, ons 3e Toppertje (in willekeurige volgorde natuurlijk), bedankt voor het klikken in de MATLAB applicatie van Jos tot je er scheel van werd, voor het pillen tellen tot je nagels er van braken, en voor de lekkere babbels en cocktails. Minetta, bedankt voor het uitwerken van de labwaardes, het meticuleus corrigeren van onze proofs, en in een ver grijs verleden je geduld met de 'spuiten'. Thijs, met jouw enthousiasme en vlotte babbel ben je een veelbelovende opvolger voor het NF1 simvastatine project (maar dat dacht ik al toen je voor het eerst je vinger opstak bij de Master's, ha!). Give it your best! Azar, our time together was short, but I really enjoyed getting to know you. I wish you all the best with your fiancée!

SP 15-45, jullie zijn haast een fenomeen! Ik heb me ontzettend welkom gevoeld in jullie kleine hokje gevuld met verhuisdozen, slingers, koekjes, pruttelende koffie en kindersurprises. Bedankt voor de fantastische sociale en statistische input. Mirjam, je was voor mij een halve paranymf en een hele steun. Petje af voor je heldere sociaal inzicht, en het managen van zo'n 21 keuze-studenten tegelijk. Ik hoop dat ik mijn kinderen in de toekomst bij jou kan brengen als ze (onverhoopt) een dokter nodig hebben. Femke, mijn hemel wat een productie en wervelende energie, ik twijfel er niet aan dat jij goed terecht komt. Is directrice van het SKZ niet iets voor jou? Idse, eigenwijze nukkige lieve behulpzame (in tegenstelling tot je bewering zélden RTFM) kerel, wanneer ga je promoveren? Ruud, zorg je als kamerjongste goed voor je roomies?

In het laatste staartje van mijn proefschrift, mede-co's: bedankt voor de gezelligheid, het delen van alle nieuwe ervaringen, en de bekers chocomelk als ik weer eens een nacht had doorgehaald!

telefoon konden kletsen over de belangrijke en totaal onbelangrijke aspecten van onderzoek en leven. Wordt vervolgd!

Graag wil ik naast mijn promotoren en co-promotor, prof.dr. W.F.M. Arts, prof.dr. B.A. Oostra, en dr. S.A. Kushner bedanken voor het plaatsnemen in de kleine commissie en het beoordelen van mijn manuscript, en Prof.dr. E. Legius, R.E. Ferner, MD FRCP, en prof.dr. F.C Verhulst voor het plaatsnemen in de grote commissie. Ik ben enorm trots dat ik mijn proefschrift mag verdedigen voor een commissie die de diversiteit van mijn werk zo goed weerspiegelt. Steven, your amazing insight and high-speed thinking generated the basis for this research. I am looking forward to your future translational projects at our university. Prof. Arts, bedankt voor het voorzitten van de kleine commissie en uw bijdrage aan de studies in dit proefschrift. Eric, ik hoop dat de Nederlands-Belgische samenwerking op klinisch en wetenschappelijk vlak blijft groeien (en dat ik me bij de verdediging niet weer vergis tussen micro-deleties en severity?). Dr. Ferner, thank you for coming to from London to join my promotion committee. De dropjes staan klaar!

Mede NF1-auteurs, door jullie verschillende achtergronden en invalshoeken heb ik van heel veel verschillende vakgebieden een graantje kunnen meepikken. Jos, aan jou had ik een zeer relaxte, positieve 4e (wat een luxe!) begeleider. Ik snap nog steeds niets van MATLAB maar gelukkig maak je prachtige applicaties voor de blondjes. Bedankt voor al je hulp met de opstelling, de theoretische achtergrond en de koffiepauzes op de goede momenten. Rianne, je bent een krachtige persoon die heel goed weet wat ze wil, en we hebben menig uurtje gediscussieerd over de ideale manier van analyseren. En verhip, ondanks het feit dat je me uiteindelijk vrij liet om de analyses naar eigen ideeën uit te voeren, zijn ze bijna zo uitgepakt als jij voorafgaand aan het onderzoek bedacht had. Hoe doe je dat....? Femke, na jaren Bourdon Vos, Rey, Beery en WISC voel ik me haast een neuropsycholoog maar ik zie ook heel duidelijk hoeveel daar nog voor nodig is. In zeer blijde verwachting gaat erom spannen of je er bij kunt zijn. Heel veel sterkte met de laatste loodjes en pufjes! Coriene, jij stond altijd klaar met goede kritische feedback en voor ruggespraak. Bedankt voor je hulp bij het interpreteren van de data (en bij de plaatjes uit de introductie!). Marlies, bedankt voor je enorme inzet tijdens het onderzoek, onze werk- en niet-werk gesprekken, en het actieve meedenken. Je staat er niet er voor niets (boven)op! Roeli, bedankt voor je sterke, lekker no-nonsense bijdrage. Ik hoop dat je een super leuk onderzoek vindt, hopelijk aan het Sophia? Saskia, dankzij jouw (zeer) geduldige uitleg werd ik een zelfstandige SPSS prof met als specialiteit multivariate regressie analyses. Jammer dat je er niet bij bent, maar ik hoop dat je het in de States heel erg naar je zin hebt met je gezin! Maarten, wat

een beetje gedesensibiliseerd is (?). Met het opzetten van het nieuwe NF1 simvastatine project en onze droom van een groot CoRe centrum heeft onze samenwerking een uitdagende toekomst!

Lieve Arja, warme, kordate, integere vrouw, alle stappen van dit onderzoek hebben we samen genomen. Alhoewel je de credits hiervoor steeds teruggeeft, hebben we samen een brug gebouwd van het Sophia naar de basale wetenschap, en het spoorboekje van onze trial doorlopen. Ik kan bijna niet beschrijven hoe belangrijk jouw niet-aflatende steun de afgelopen jaren voor mij is geweest. Hoe vaak heb jij me niet gebeld om te vragen hoe het ging? Voor mij een eye-opener, maar voor jou overduidelijk, is dat de belangrijkste les die ik van jou kan leren is, hoe je hart voor je werk kunt combineren met een ontspannend en vervullend persoonlijk leven. Daarnaast heb je me laten zien hoe ontzettend mooi het is om een goede, betrokken arts te zijn, en kreeg ik de kans om te ervaren hoezeer ik mij daarin in mijn element voel. Het is voor mij niet meer dan vanzelfsprekend dat jij op de grote dag als paranimf naast me staat. Ik ben blij dat je, na de eerste schrik, hiertoe bereid bent.

Henriëtte, onze samenwerking telt een groot aantal prachtige hoogtepunten en daarmee samengaand, zoals je ze zelf benoemde toen je mijn 'Brief 2' op de motorkap van je auto tekende, heel wat ludieke ondernemingen. Ik ben erg blij dat ik tijdens het laatste deel van ons project op jouw afdeling mocht werken, in een warm team onder jouw krachtige, doelmatige (ook een toepasselijk woord) leiding. Zo heb ik een essentieel stuk basisvaardigheden voor statistisch en klinisch verantwoord medisch onderzoek kunnen oppikken (een mens is nu eenmaal geen muis!). Henriette, je bent een zeldzaam integer mens, doortastend en daadkrachtig, met op alle problemen een heldere en creatieve oplossing. Geen wonder dat je met deze kwaliteiten professor bent geworden! Ik ben er trots op jou als promotor te hebben.

Chris, bedankt voor het begeleiden van mijn promotie. Je bent een leider met visie, en je vermogen om mensen te enthousiasmeren om het onderzoek in te duiken en keihard te werken om er het uiterste uit te halen heb ik aan den lijve ondervonden. Ik ben benieuwd of we in het vervolgonderzoek kinderen kunnen leren fietsen. Misschien komen we dan nog een keer op tv?

Susan, paranimf, Toppertje met een keihoog IQ én EQ, rationeel met bij tijd en wijle een lekkere portie weifelkonterigheid.... Tijdens onze labjaren volgde onze emotionele conjunctuur gek genoeg vaak een volledig in-fase- of juist precies uit-fase patroon. Ik ben blij dat we altijd bij elkaar terecht konden en dat we uren en uren op het lab, voor de metro-ingang, of aan de

Dankwoord

Bij het uitvoeren van dit promotie onderzoek ben ik mijn sterkste, meest onvermoede en, ja, ook mijn minst sterke kanten tegen gekomen. Hierbij heb ik steun en inspiratie ontvangen van heel veel verschillende mensen (bijkomend voordeel van multidisciplinair onderzoek), die allemaal een stukje hebben bijgedragen aan mijn groei en ontwikkeling. Hier wil ik deze voor mij belangrijke personen bedanken.

Allereerst gaat mijn dank uit naar alle kinderen en ouders die mee hebben gedaan aan ons onderzoek, voor hun hartverwarmende inzet en enthousiasme. Daarnaast wil ik de NF Vereniging Nederland bedanken voor hun belangrijke bijdragen om het onderzoek naar de cognitieve problemen bij NF1 te bevorderen. Zonder jullie geen onderzoek!

Ype, mijn co-promotor, zonder jou zou ik niet staan waar ik nu sta. Begin 2002 startte ik als piepjonge Neuroscience Master student in jouw – ook nog piepjonge – onderzoeksgroep. Ik begon vol enthousiasme op het lab, maar toen ik de pipet nog steeds spuit bleef noemen, en van hele muizenfamilies perfuseren behoorlijk ongelukkig werd, was mijn carrière bijna heel anders gelopen. In een vlaag van de creatieve hyperactiviteit waar jouw brein om bekend staat (en die de toon zette voor nog veel meer onderzoek) bedacht je een volledig nieuw project, waarin ik me als een vis in het water voelde: onderzoek naar de cognitieve problemen bij kinderen met NF1. Een week later liep ik met Arja mijn eerste NF1 spreekuur. Toen we hoorden dat jouw vorige lab erin geslaagd was het coginitief fenotype van *Nf1* muizen om te keren met behulp van een veilige, direct toepasbare therapie, was nu of nooit. We spraken de magische woorden "dit moeten we doen" en "dat is goed", en mijn promotie-onderzoek was geboren (met de bedenkers nog 'blissfully ignorant' van wat er allemaal komt kijken bij het van de grond af opzetten van zo'n enorm project...).

Voor mij was het balanceren op het grensvlak tussen basale wetenschap en kliniek een enorm waardevolle ervaring. De contrasten tussen rationele onderzoeksminded-heid en klinisch denken, snelle beslissingen maken en statistiek vooráf, flexibele samenwerking en commissies, en 'evidence based' en 'ervaringsdeskundigheid' maakte het ons allebei niet altijd makkelijk. We waren het lang niet altijd met elkaar eens, en onze discussies (tussen een Fries en een kwart-Fries) zijn berucht. Ype, ik hoop dat ik een stukje van je inventiviteit en doorzettingsvermogen, die jou zo ontzettend ver hebben gebracht, mee kan nemen in mijn toekomstige ondernemingen. Ik hoop ook dat, in het kader van de kruisbestuiving, je witte jassen-allergie al

CHAPTER 10

PERSONAL NOTES:

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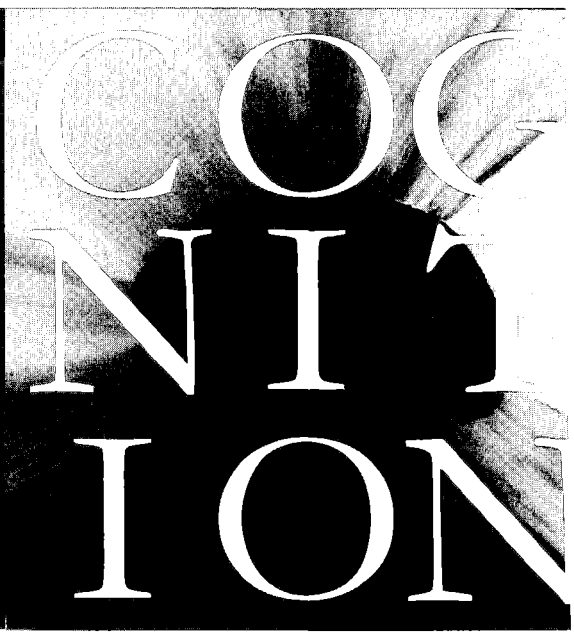

vervolg trials nodig om te uit te vinden of lange-termijn behandeling met statines de problemen op school, met gedrag en in Kwaliteit van Leven van kinderen met NF1 kan verbeteren.

Er werd geen effect van simvastatine gevonden op de primaire uitkomstmaten (Rey Complexe Figuur – lange termijn, de Cancellation test – snelheid, prisma adaptatie en de gemiddelde brein ADC-waarde. In de secundaire uitkomstmaten vonden we een significant betere score in de simvastatine groep op Figuur Leggen (β=0.54, 95% confidentie interval [CI] 0.08-1.01). Deze verbetering was met name zichtbaar bij de kinderen met een lage uitgangs score op deze test (β=0.80, 95% CI: 0.29-1.30). Deze verbetering in Figuur Leggen kan echter een toevalsbevinding zijn, en de uiteindelijke conclusie van deze studie was dat een korte-termijn behandeling met simvasatatine geen verbetering teweeg brengt in het cognitief functioneren van NF1 patiënten.

Ondanks het feit dat korte-termijn behandeling met simvastatine geen verbetering van het cognitief functioneren van kinderen met NF1 lijkt te bewerkstelligen, vraagt het goede veiligheids-profiel van statines om vervolg-onderzoek. In **hoofdstuk 8** hebben we verscheidene beperkingen van onze studie besproken, die mogelijk het vinden van een effect kunnen hebben bemoeilijkt. Daarnaast hebben we aanbevelingen gegeven voor vervolg-onderzoek. De belangrijkste aanbeveling is om kinderen voor een langere periode te behandelen. Een langere behandelingsduur zou mogelijk het placebo-effect uitdoven, leer-effecten verminderen, geeft ruimte voor een langere blootstelling aan de hoogste therapeutische dosis, en geeft het brein langer de tijd om zijn functie te herstellen. Daarnaast maakt een langere behandelingsduur het mogelijk om uitkomstmaten uit het dagelijks leven mee te nemen, zoals het functioneren op school, gedrag en Kwaliteit van Leven. Een vervolg-trial met een behandelingsduur van 1 jaar is op dit moment in voorbereideng aan het Erasmus MC – Sophia Kinderziekenhuis Rotterdam.

In **hoofdstuk 8** concluderen we dat NF1 een grote impact heeft op het dagelijks functioneren, omdat kinderen grote problemen hebben op school, met motoriek en, althans volgens ouders, een lagere Kwaliteit van Leven hebben. Kennis van de problemen die kunnen vóórkomen bij NF1 kan mogelijk een tijdige herkenning en adequate interventie faciliteren. Onze onderzoeken hebben een aantal potentiële gebieden voor structurele ondersteuning aangewezen, zoals gedragsproblemen. Daarnaast hebben we een aantal uitkomstmaten geïdentificeerd die gebruikt kunnen worden op potentiële nieuwe behandelmethodes voor cognitieve problemen bij NF1 patiënten te evalueren.

Alhoewel de preklinische studies hoopgevend waren, bewerkstelligt een korte-termijn behandeling met statines geen verbetering in het cognitief functioneren. Desalniettemin zijn

adaptatie van handbewegingen. De laatste twee testen doen een beroep op de capaciteit tot motor-leren, waarbij met name het cerebellum een rol speelt.

Hoewel we konden bevestigen dat kinderen met NF1 problemen hebben met visuo-motor integratie en fijne motoriek, zagen we geen significante afwijkingen in de coördinatie van oog- of handbewegingen, of in de adaptatie van saccadische oogbewegingen. Wel lieten kinderen met NF1 afwijkingen zien in de prisma-geïnduceerde adaptatie van handbewegingen. Onze resultaten suggereren dat de problemen in de motoriek die kinderen met NF1 in het dagelijks leven ervaren deels gerelateerd kunnen zijn aan stoornissen in het motor-leren. Deze afwijkingen lijken te worden veroorzaakt door problemen in specifieke subregio's van het cerebrum en het cerebellum, maar niet door een volledig dysfunctioneren van deze hersengebieden.

De meerderheid van de kinderen met NF1 laat op T2-gewogen MRI opnames van het brein hyperintensiteiten zien, zogenaamde Unidentified Bright Objects (ongeïdentificeerde heldere objecten; UBOs). We onderzochten de aard van deze UBOs door bij kinderen met NF1 en gezonde controles Diffusie-gewogen opnames te maken van 7 vooraf gedefiniëerde hersengebieden, waaronder de gebieden die het meest aangedaan zijn door UBOs **(hoofdstuk 6)**. We observeerden een hogere Apparent Diffusion Content (ADC-waardes) in de hersengebieden aangedaan door UBOs vergeleken met de hersengebieden waar geen UBO aanwezig was, in kinderen met NF1. Daarnaast waren de ADC-waardes in kinderen met NF1 ook in de niet door een UBO aangedane gebieden hoger dan in controles. Deze verhoogde ADC-waardes wijzen op een verhoogde totale hoeveelheid water in het hersen parenchym van NF1 patiënten. Door eigenvalues uit Diffusie-tensor opnames te onderzoeken vonden we een indicatie dat dat deze water accumulatie zich in eerder in de myeline schede dan in axonen bevindt.

In **hoofdstuk 7** evalueerden we het effect van simvastatine op het cognitief functioneren, motor leren en hersen-afwijkingen van kinderen met NF1 in een gerandomiseerde, placebo-gecontroleerde, dubbel-blinde trial. 62 kinderen met NF1 van 8 tot en met 16 jaar werden gedurende 12 weken één maal daags behandeld met simvastatine of een placebo. Het effect van simvastatine werd onderzocht met neuropsychologische, neurofysiologische en neuroradiologische uitkomstmaten, waarvan een deel werd geïdentificeerd in de onderzoeken in **hoofdstuk 3, 5 en 6.**

van problemen met het functioneren op school, waarbij in ieder geval 75% van de kinderen meer dan één standaard deviatie achterloopt bij hun klasgenoten. Daarnaast krijgt de meerderheid van de kinderen extra ondersteuning, in de vorm van speciaal onderwijs (40%), of remedial teaching voor (een combinatie van) problemen met leren, motoriek, spraak en gedrag (in totaal 85%). Een belangrijke bevinding was dat de groep jonge kinderen die geen evidente problemen in de schoolprestaties heeft, mogelijk risico loopt op het ontwikkelen van leerproblemen, omdat zij substantiële neuropsychologische stoornissen laten zien. Tenslotte werd er een sterke relatie gevonden tussen cognitie en de klinische ernst van NF1. Kinderen met ernstiger klinische tekenen van NF1 hadden meer problemen met het cognitief functioneren en slechtere schoolprestaties. Deze bevindingen geven een duidelijk beeld van de grote impact van NF1 op de schoolprestaties.

In **hoofdstuk 4** werd de Gezondheid Gerelateerde Kwaliteit van Leven bij kinderen met NF1 onderzocht met behulp van vragenlijsten ingevuld door ouders, en door de kinderen zelf. Daarnaast onderzochten we de potentiële bijdrage van demografische factoren, ziekte-specifieke factoren, en problemen met het functioneren op school en het gedrag, aan de Gezondheid Gerelateerde Kwaliteit van Leven. Ouders rapporteerden een substantiële impact van NF1 op 9 van de 13 Gezondheid Gerelateerde Kwaliteit van Leven domeinen, die problemen weerspiegelen op het fysieke, sociale, emotionele en gedrags vlak. In tegenstelling tot hun ouders rapporteerden kinderen met NF1 alleen problemen in het domein Lichamelijke Pijn.

Een onverwachte bevinding van ons onderzoek naar Kwaliteit van Leven was dat ondanks het feit dat we uitgebreide schoolproblemen zagen in onze onderzoekspopulatie, deze problemen niet lijken bij te dragen aan de Gezondheid Gerelateerde Kwaliteit van Leven scores van ouders en kinderen. Gedragsproblemen (gescoord door leerkrachten) zijn echter duidelijk geassocieerd met Gezondheid Gerelateerde Kwaliteit van Leven-scores van ouders. Dit wijst op een een interessante mogelijkheid om niet alleen de gedragsproblemen zelf, maar ook de algemene Kwaliteit van Leven van kinderen met NF1 te verbeteren door gedragsproblemen aan te pakken.

Een groot deel van de kinderen met NF1 heeft problemen met de fijne of grove motoriek. In **hoofdstuk 5** onderzochten we de motorische vaardigheden van kinderen met NF1 en gezonde kinderen met een test voor fijne motoriek en visuo-motorische integratie, en paradigma's voor de adaptatie (het leren aanpassen) van saccadische oogbewegingen en prisma-geïnduceerde

Nederlandse Samenvatting

Achtergrond (hoofdstuk 1)

Neurofibromatose type 1 (NF1) is een autosomaal dominante ziekte, veroorzaakt door een heterozygote mutatie in het gen voor het eiwit neurofibromine. NF1 kan invloed hebben op het lichamelijk functioneren en het uiterlijk, maar ook op het cognitief functioneren. De cognitieve problemen bij NF1 behelzen onder andere neuropsychologische stoornissen, en problemen met leren, gedrag, en motorische vaardigheden. Deze cognitieve problemen zijn de meest voorkomende complicatie van NF1 op de kinderleeftijd.

Onderzoek in muizen met een heterozygote *Nf1* deletie toont aan dat de het cognitief fenotype van NF1 wordt veroozaakt door verhoogde activiteit van de RAS/ERK signaal transductie route. Een zeer belangrijke bevinding is dat behandeling met statines (cholesterol verlagende middelen) de verhoogde RAS activiteit in *Nf1* muizen kan terugbrengen, en hun verstoorde synaptische plasticiteit, en problemen met leren en geheugen en aandacht kan verhelpen. Omdat statines zo effectief zijn in muizen, en een zeer goed veiligheids-profiel hebben, zijn ze een ideaal potentieel medicijn om de cognitieve stoornissen van NF1 patiënten te behandelen.

De doelen van het onderzoek in dit proefschrift waren het verkrijgen van inzicht in de impact van NF1 op het dagelijks leven, het identificeren van mogelijke uitkomstmaten die kunnen worden gebruikt om het effect van potentiële therapeutische interventies te evalueren, en het onderzoeken van het effect van simvastatine op de cognitieve problemen van kinderen met NF1 in een gerandomiseerde, dubbel-blinde, placebo-gecontroleerde trial.

In **hoofdstuk 2** werd een overzicht gegeven van de huidige kennis van de etiologie van de cognitieve problemen bij NF1 en gerelateerde aandoeningen binnen de neuro-cardio-facio-cutane en Hamartoma syndromen, en werden potentiële behandelingsstrategieën besproken die uit oncologisch onderzoek en uit onderzoek met diermodellen naar voren zijn gekomen.

Hoewel verscheidene studies laten zien dat NF1 patiënten problemen hebben met taken voor specifieke neuropsychologische domeinen en op testen voor academische voortgang, was er niet veel bekend over hoe deze problemen vertaald worden in het functioneren op school. Om hier inzicht in te krijgen hebben we naast formeel neuropsychologisch onderzoek, een inventaris gemaakt van de schoolprestaties van een grote groep kinderen met NF1, zoals beschreven in **hoofdstuk 3.** We hebben aangetoond dat er onder kinderen met NF1 een hoge prevalentie is

attenuate the placebo effect, wear off practice effects, enable a longer exposure to the highest therapeutic dose, and allow for a longer time for the brain to restore function. Moreover, a longer treatment duration would allow inclusion of real-life outcome measures such as school performance, behavior and quality of life. A follow-up trial with a treatment period of 1 year is currently in preparation at the Erasmus MC – Sophia Children's Hospital Rotterdam.

In **chapter 8**, we conclude that NF1 has a large impact on daily life functioning, as NF1 children show substantial problems in school performance and motor functioning and, at least according to parents, have a lower quality of life. Awareness of the problems associated with NF1 may facilitate timely recognition and appropriate intervention. Our studies have pointed out several potential targets for structural support, such as behavioral problems. Also, we have identified several outcome measures that can be used to assess potential treatments for cognitive deficits in NF1.

Although preclinical studies were promising, short-term statin treatment did not improve cognitive functioning. Still, further clinical trials are needed to reveal whether long-term statin treatment can improve school performance, behavioral problems, and quality of life in children with NF1.

within specific regions of the cerebellum and cerebrum, but not by a ubiquitous malfunctioning of these brain regions as a whole.

The majority of children with NF1 display hyperintensities on T2-weighed MRI of the brain, so-called UBOs (Unidentified Bright Objects). We examined the nature of UBOs by performing Diffusion-weighed Imaging of 7 predetermined brain regions, including those predominantly affected by UBOs, in children with NF1 and controls **(chapter 6)**. We observed increased Apparent Diffusion Content (ADC values) in UBO-affected brain areas compared to UBO-unaffected areas of NF1 children. In addition, ADC values were higher in NF1 children than in controls, also in UBO-unaffected brain areas. These elevated ADC values indicate increased overall water content in NF1 brain parenchyma. By examining eigenvalues obtained with Diffusion Tensor Imaging we found evidence that this fluid accumulation is intra-myelinic rather than axonal.

In **chapter 7,** we assessed the effect of simvastatin on cognitive performance, motor learning and brain abnormalities in children with NF1 in a randomized, placebo-controlled, double blind trial. 62 Children with NF1 aged 8 to 16 years were treated with simvastatin or placebo once a day for 12 weeks. The effect of simvastatin was assessed using neuropsychological, neurophysiological and neuroradiological outcome parameters, part of which were identified in **chapters 3, 5 and 6.**

We did not find an effect of simvastatin on the primary outcome measures (Rey Complex Figure test [delayed recall], Cancellation test [speed], Prism Adaptation and average brain Apparent Diffusion Coefficient). On the secondary outcome measures, we found a significant improvement in the simvastatin group in object assembly scores (ß=0.54, Confidence Interval [CI]: 0.08–1.01), which was specifically observed in children with poor baseline performance (ß=0.80, CI: 0.29–1.30). However, the results on object assembly could be a spurious finding, and the overall conclusion of this study was that short-term simvastatin treatment does not improve cognitive functioning in NF1 patients.

Despite the fact that short-term treatment did not reveal an effect of simvastatin on cognitive functioning in NF1 children, the favorable safety profile of statins does call for follow-up studies. In **chapter 8**, we pointed out several limitations of our study that could have hampered identification of an effect, and give recommendations for future trials. The major recommendation for follow-up studies is to treat children for a longer period. This may

found that the young children that did not have evident problems in school functioning were potentially at risk for developing learning disabilities, as they frequently did display substantial neuropsychological deficits. Lastly, we observed a clear relationship between cognitive performance and clinical severity of NF1. Children with more severe clinical signs of NF1 were more impaired in their cognitive functioning and their school functioning. In all, this study clearly illustrates the large impact of NF1 on school performance.

In **chapter 4**, we assessed Health Related Quality of Life in children with NF1 using parental reports and children's self-reports, and investigated the potential contribution of demographic factors, disease-specific factors, and problems in school performance or behavior. Parents report a profound impact of NF1 on 9 out of 13 Health Related Quality of Life domains, reflecting difficulties in physical, social, behavioral and emotional aspects of quality of life. In contrast, children themselves only reported problems on Bodily Pain.

An unexpected finding of our study on Quality of Life was that despite the fact that we found extensive problems in school performance in our population, these problems do not seem to contribute to Health Related Quality of Life scores of parents or children. Importantly, we revealed that behavioral problems (rated by teachers) are a prominent predictor of parent Health Related Quality of Life scores. This points to an exciting potential opportunity to improve not only behavioral problems but also overall quality of life in children with NF1 by addressing these behavioral problems.

Children with NF1 frequently display problems in fine and gross motor functioning. In **chapter 5,** we examined motor performance in children with NF1 and controls, using a test for fine motor performance and visual-motor integration, and paradigms for saccadic eye movement adaptation and prism-induced hand movement adaptation. The latter two tests assess motor learning capacities controlled by mainly cerebellar processing.

Although we confirmed that NF1 children have problems in visual-motor integration and fine motor coordination, we did not observe significant impairments in motor performance of either eye or arm movements, or adaptation of saccadic eye movements. However, NF1 children did show deficits in motor learning during prism-induced hand movement adaptation. Taken together, our results suggest that the motor problems of children with NF1 in daily life may partly be related to deficits in motor learning. These deficits may be caused by aberrations

Summary

Background (chapter 1)

Neurofibromatosis type 1 (NF1) is an autosomal dominant neurocutaneous disease, caused by a heterozygous mutation in the gene encoding the neurofibromin protein. NF1 can affect physical functioning and appearance, as well as cognitive performance. The cognitive deficits of NF1 include neuropsychological deficits, learning disabilities, behavioral problems, and motor problems, and are considered to be the most common complication at pediatric age.

Studies in heterozygous *Nf1* knockout mice have revealed that the cognitive phenotype of NF1 is caused by elevated activity of the RAS/ERK signal transduction pathway. Excitingly, treatment with statins (cholesterol lowering drugs) can reverse the increased RAS activity in *Nf1* mice, and rescue their deficits in synaptic plasticity, learning and memory, and attention. The fact that statins are effective in *Nf1* mice, combined with their very good safety profile, makes them an ideal candidate drug to treat cognitive impairments associated with NF1 in patients.

The overall objectives of this thesis were to provide an overview of the impact of NF1 on daily life, to identify possible outcome measures that can be used to assess potential therapeutic interventions, and to investigate the effect of simvastatin on cognitive problems in NF1 using a randomized, double-blind, placebo controlled trial.

In **chapter 2,** we reviewed the current knowledge of the etiology of cognitive deficits in NF1 and related disorders within the neuro-cardio-facial-cutaneous and Hamartoma syndromes, and gave an overview of potential treatment options that were found using knowledge from the oncology field and studies on animal models.

Although it was known from other studies that NF1 patients have impairments in specific neuropsychological domains and in academic achievement tests, less was known about the impact of these impairments on school performance. Therefore, in addition to formal neuropsychological assessment, we inventorized school performance in a large group of NF1 patients, as described in **chapter 3**. We uncovered that problems in school performance are highly prevalent among children with NF1, with at least 75% of the children lagging more than 1 standard deviation behind grade peers. In addition, the majority of children received additional support, in the form of special education (40%), or remedial teaching for (a combination of) problems in learning, motor functioning, speech and behavior (85% in total). Importantly, we

CHAPTER 9

Summary – Samenvatting

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