Original Article

A clinical and genetic overview of 18 years neurofibromatosis type 1 molecular diagnostics in the Netherlands


NF1 mutations are the underlying cause of neurofibromatosis type 1 (NF1), a neuro-cardio-facio-cutaneous syndrome (NCFC). Because of the clinical overlap between NCFCs, genetic analysis of NF1 is necessary to confirm a clinical diagnosis NF1. This report describes the clinical and genetic findings of 18 years of NF1 molecular diagnostics in the Netherlands. A pathogenic mutation was found in 59.3% (1178/1985) of the index patients, mostly de novo (73.8%). The majority of the index patients (64.3%) fulfilled the National Institute of Health NF1 criteria, a pathogenic mutation was found in 80.9% of these patients. Seventy-four percent of the index patients with an NF1 pathogenic mutation and not fulfilling the NF1 criteria is <12 years, in agreement with the fact that some NF1 symptoms appear after puberty. Genotype–phenotype correlations were studied for 527 index patients. NF1 patients with a type 1 microdeletion have a sixfold higher risk of special education vs NF1 patients with an intragenic mutation. No evidently milder NF1 phenotype for patients with a missense mutation was observed. Forty-six prenatal analyses were performed in 28 (2.4%) families, of which 29 (63%) showed heterozygosity for the familial pathogenic mutation. This indicates that there is a need for prenatal NF1 testing.

Conflict of interest
The authors declare no conflict of interest.

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder that is part of the neuro-cardio-facio-cutaneous syndromes (NCFCs) or RASopathies, a group of disorders in which the nervous system, circulatory system, craniofacial and cutaneous development is affected (1, 2). NF1 is a common genetic disorder with a birth incidence of about 1:3000 (3, 4), half of which are sporadic cases (5). The NF1 phenotype is highly variable and characteristics develop over time (5, 6). The main clinical features of NF1 are café au lait (CAL) spots, skin fold freckling, cutaneous neurofibromas and Lisch nodules. Minor features include short stature, mild macrocephaly and learning difficulties (6, 7).

A diagnosis of NF1 is clinically confirmed by the presence of two or more of the following diagnostic criteria as formulated by the National Institute of Health (NIH) in 1988; six or more CAL macules (>5 mm in children or >15 mm adults), two or more cutaneous/subcutaneous neurofibromas or one plexiform neurofibroma, freckling in the axillary or inguinal regions, optic glioma, two or more Lisch nodules,
bony dysplasia (sphenoid wing dysplasia and bowing of long bone pseudoarthrosis), first degree relative with NF1 (8, 9).

NF1 is caused by mutations in NF1, the tumor suppressor gene that encodes the cytoplasmatic protein neurofibromin. Neurofibromin is a ras guanosine triphosphatase (GTPase) activating protein that inhibits Ras signaling and as such acts as a regulator of signaling for cell proliferation and differentiation. Mutations in NF1 result in loss of function of neurofibromin causing an increase in Ras signaling (10). Because of the clinical overlap between the NCFCs (e.g. Legius syndrome), genetic analysis of NF1 is necessary to confirm the clinical diagnosis. If a pathogenic mutation is identified, genetic testing of family members can be offered (including prenatal analysis).

Clear genotype–phenotype correlations have so far been reported only in two cases. It has been shown that NF1 patients with an NF1 microdeletion have a more severe clinical phenotype, including a higher prevalence of learning disabilities and dysmorphic features, compared to patients with an intragenic NF1 mutation (11–13). Another observation is that NF1 patients heterozygous for the 3-bp inframe deletion c.2970_2972delAAT in NF1 all lack cutaneous neurofibromas (14). Other genotype–phenotype correlations have been reported but further investigations are needed to confirm these observations [reviewed in reference (15)].

In this report, an overview is given about the genetic analysis of NF1 in the Netherlands over a period of 18 years. Both the clinical and genetic aspects of NF1 molecular diagnostics are described.

Subjects and methods

Study population

The study population consisted of index patients of 1985 families whose samples were sent to the DNA diagnostic laboratory of our department between 1993 and 2010 for genetic analysis of NF1. Samples were sent by clinical geneticists (n = 1174, 57.5%), pediatricians (n = 500, 24.5%), (pediatric) neurologists (n = 161, 7.9%), dermatologists (n = 12, 0.6%), or other medical specialists (n = 195, 9.5%) because of a clinical diagnosis or symptoms of NF1. The mean age of patients at the time of blood sampling was 21.3 years (range 0–83). Males (50.2%) and females (49.8%) were equally represented. Written informed consent was obtained from all patients.

Mutation analysis

DNA was isolated from peripheral blood cells or chorionic villus samples according to standard procedures. During the 18 years of NF1 molecular diagnostics, several techniques were used to analyze NF1 for genetic variants (see Fig. S1). From 1993 till 2002, Fluorescence in situ hybridization (FISH) analysis and single strand conformation polymorphism (SSCP) analysis were used. Aberrant SSCP patterns were followed by Sanger sequencing. Since 2003 Sanger sequencing was performed as the initial testing method in combination with FISH analysis (2003–2006) or with multiplex ligation-dependent probe amplification (MLPA; starting in 2007). Sanger sequencing was also used, respectively, to test index patients negative for NF1 mutations using SSCP in the period 1993–2002. SSCP and FISH analyses were performed as described before (16–18). For FISH analysis, the probe combinations RP11-14206 and RP11-252O24 or RP11-451O17 and CTD-2370N5 were used. Sanger sequencing was performed on an ABI 3730XL automated sequencer (Applied Biosystems, Foster City, CA) and all coding exons and exon/intron boundaries (30 bp into the introns) of NF1 (NM_000267.3, exons 1–58, excluding alternative exon 31) and SPRED1 (NM_152594.2, exons 1–7) were analyzed (primers available on request). SALSA MLPA kits P081/82 and P295 (MRC Holland, Amsterdam, the Netherlands) were used to detect large rearrangements in, respectively, NF1 and SPRED1. SALSA MLPA kit P122-C2 was used to determine the type of NF1 microdeletion if a total NF1 deletion was found. MLPA analysis was performed according to the manufacturer’s instructions. MLPA products were run on an ABI 3730XL automated sequencer and data were analyzed using Genemarker software version 1.5 (Softgenetics, State College, PA). Total or almost total deletions of NF1 were confirmed by FISH analysis. The software program Alamut version 2 (Interactive Biosoftware, Rouen, France) was used to help with the interpretation of sequence variants (e.g. splice site prediction, see Appendix S1, using Alamut). If a sequence change of unknown clinical significance was identified in the index patient, parents were analyzed and paternity testing was performed. If parents tested negative, the sequence change was classified as pathogenic. Paternity testing was performed using the Identifier kit (Applied Biosystems). This kit was also used for testing the fetal origin of the DNA during prenatal testing. All sequence variants have been deposited in a Leiden Open Variation Database (LOVD) database (19), accessible at http://www.lovd.nl/NF1.

Clinical data

Detailed information about the clinical symptoms of the patients was obtained either during the consults of a clinical geneticist of our department (internal approach) or by sending a questionnaire (available on request) to the referring physician (outside the Erasmus Medical Centre) of the index patients [external approach; sent between 20 October 2005 till 31 December 2010 (n = 1048)]. The questionnaires included items such as occurrence of clinical symptoms of NF1 according to the NIH criteria, the occurrence of tumors (e.g. neurinoma, meningioma) and whether other (neurological) problems were present. Questionnaires were sent together with the results of the initial NF1 analysis of the index patient. Reminders were only sent in case parents were tested for the presence/absence of
the sequence change identified in their child. Clinical data of 821 index patients was obtained [306 during a consult and 515 via a questionnaire (response rate = 49.1%)].

Data analysis

Clinical data was stored in an in-house developed Access database, which was coupled to the Erasmus Medical Centre patient information system and to the patient information system of the Department of Clinical Genetics. A chi-squared test was used to test for differences in distribution between two categories (e.g. external and internal index patients). Short stature is defined as length ≤2 SD below the age- and sex-matched population mean. Macrocephaly is defined as head circumference ≥2 SD above the age- and sex-matched population mean. Genotype–phenotype correlations were studied using a multiple logistic regression model with the covariates sex and age at time of blood sampling to control for potential confounding. Index patients with a pathogenic mutation (and clinical data) were included (n = 527). Major and minor features (Tables 1 and 2) were tested individually with the type of mutation (e.g. microdeletion vs intragenic mutation). Odds ratios (OR) and 95% confidence intervals (CI) were calculated when there was a significant effect. The Bonferroni method was used to correct for multiple testing [significant level is p = 0.003 (0.05 divided by 18 clinical features)]. Statistical analyses were performed with the software program IBM SPSS STATISTIC version 20 (SPSS Inc, Chicago, IL).

Results

A total of 1985 index patients were tested for mutations in NF1 for the last 18 years, starting with <50 patients/year in the early years and increasing to >250 patients in 2010 (Fig. 1). The mutation detection rate (average = 63.3%) was stable over the years. In total, a pathogenic mutation was identified in 1178 patients

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**Table 1. Major NF1 criteria present in index patients**

<table>
<thead>
<tr>
<th>Major criteria</th>
<th>Internal index patients (n = 306)</th>
<th>External index patients (n = 515)</th>
<th>Index patients overall (n = 821)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fulfilled NF1 criteria (n = 231)</td>
<td>Not fulfilled NF1 criteria (n = 75)</td>
<td>Fulfilled NF1 criteria (n = 297)</td>
</tr>
<tr>
<td>Multiple café au lait spots</td>
<td>219 (94.8)</td>
<td>31 (41.3)</td>
<td>268 (90.2)</td>
</tr>
<tr>
<td>Freckling</td>
<td>187 (81.0)</td>
<td>4 (5.3)</td>
<td>248 (83.2)</td>
</tr>
<tr>
<td>Multiple neurofibromas</td>
<td>111 (48.1)</td>
<td>12 (16.0)</td>
<td>154 (51.9)</td>
</tr>
<tr>
<td>Plexiform neurofibroma</td>
<td>67 (29.0)</td>
<td>2 (2.7)</td>
<td>48 (16.2)</td>
</tr>
<tr>
<td>Lisch nodules</td>
<td>70 (30.3)</td>
<td>0 (0.0)</td>
<td>133 (44.8)</td>
</tr>
<tr>
<td>Optic glioma</td>
<td>17 (7.4)</td>
<td>2 (2.7)</td>
<td>19 (6.4)</td>
</tr>
<tr>
<td>Bony dysplasia</td>
<td>13 (5.6)</td>
<td>1 (1.3)</td>
<td>14 (4.7)</td>
</tr>
<tr>
<td>Positive family history NF1</td>
<td>47 (20.3)</td>
<td>0 (0.0)</td>
<td>16 (5.4)</td>
</tr>
</tbody>
</table>

**Table 2. Minor NF1 features present in index patients**

<table>
<thead>
<tr>
<th>Minor features</th>
<th>Internal index patients (n = 306)</th>
<th>External index patients (n = 515)</th>
<th>Index patients overall (n = 821)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fulfilled NF1 criteria (n = 231)</td>
<td>Not fulfilled NF1 criteria (n = 75)</td>
<td>Fulfilled NF1 criteria (n = 297)</td>
</tr>
<tr>
<td>Tumors a</td>
<td>20 (8.7)</td>
<td>10 (13.3)</td>
<td>22 (7.4)</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>25 (10.8)</td>
<td>9 (12.0)</td>
<td>41 (13.8)</td>
</tr>
<tr>
<td>Macrocephaly</td>
<td>30 (13.0)</td>
<td>4 (5.3)</td>
<td>37 (12.5)</td>
</tr>
<tr>
<td>Short stature</td>
<td>21 (9.1)</td>
<td>3 (4.0)</td>
<td>4 (1.3)</td>
</tr>
<tr>
<td>Learning problems</td>
<td>55 (23.8)</td>
<td>7 (9.3)</td>
<td>105 (35.4)</td>
</tr>
<tr>
<td>Special education</td>
<td>47 (20.3)</td>
<td>8 (10.7)</td>
<td>58 (19.5)</td>
</tr>
<tr>
<td>Mental retardation</td>
<td>12 (5.2)</td>
<td>3 (4.0)</td>
<td>16 (5.4)</td>
</tr>
<tr>
<td>Speaking problems</td>
<td>65 (28.1)</td>
<td>3 (4.0)</td>
<td>23 (7.7)</td>
</tr>
<tr>
<td>Behavior problems</td>
<td>42 (18.2)</td>
<td>5 (6.7)</td>
<td>16 (5.4)</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>44 (17.7)</td>
<td>1 (1.3)</td>
<td>14 (4.7)</td>
</tr>
<tr>
<td>No minor feature</td>
<td>76 (32.9)</td>
<td>41 (54.7)</td>
<td>136 (46.8)</td>
</tr>
</tbody>
</table>

aExcluding tumors described in Table 1. A full list of the different types of tumors (n = 24) observed in the index patients is available in Table S1.
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(59.3%), an unclassified variant (UV) in 7.3% of the index patients and no pathogenic mutation or UV was detected in the remaining patients (33.4%) (Fig. 1). A slightly different distribution of sequence variants was found in the group of index patients with clinical data \( (n = 821; \text{pathogenic mutations 64.2\%, UVs 8.2\%}) \) and no UVs or pathogenic mutations \( 27.6\%, \ p = 0.011 \) (Table S1). Of the index patients with a pathogenic mutation \( (n = 1178) \), parents were tested of 432 cases. Of these, 319 index patients (73.8%) appeared to have a \textit{de novo} mutation and 113 cases were familial (26.2%). The proportion \textit{de novo} vs familial mutation was nearly the same when compared with the information obtained via consult or questionnaire (73.7% sporadic vs 26.3% familial). Pathogenic mutations identified in index patients were distributed as follows: frameshift variants (31.7%), nonsense variants (29.4%), splicing variants (19.7%), missense variants (9.8%), \textit{NF1} microdeletions (5.3%) and a group of other mutations types (e.g. in frame events; 4.1%) (Table S4). For frequencies of the different types of pathogenic mutations for index patients with and without clinical data, see Fig. S2. The distribution of the type of pathogenic mutations was similar between the different patient groups. Sequence variants were, in agreement with previous studies, distributed along \textit{NF1} without any clear hotspots (Figs S3a,b) (20, 21).

The presence of \textit{NF1} major clinical criteria in the index patients is shown in Fig. 2. The majority of the index patients fulfilled the NIH criteria for \textit{NF1} (average: 64.3\%, internal index patients: 75.5\%, external index patients: 57.7\%). A significant difference in distribution was found between the internal and external collected data of the index patients \( (p = 4.8 \times 10^{-5}). \)

An overview of the different major clinical criteria of \textit{NF1} present in the index patients is given in Table 1. There was no significant difference in distribution of the major \textit{NF1} criteria between the internal and external collected index patients not fulfilling the \textit{NF1} criteria \( (p > 0.1) \), whereas the opposite was the case for index patients fulfilling the \textit{NF1} criteria \( (p = 6.5 \times 10^{-7}). \) This difference in distribution is mainly caused by the higher percentage of plexiform neurofibromas (29.0%) and positive \textit{NF1} family history (20.3%) and a lower percentage of Lisch nodules (30.3%) in the internal collected index patients vs that found in the external collected index patients (16.2\%, 4\% and 44.8\%, respectively).

In Fig. 3, the distribution of genetic variants is displayed for index patients fulfilling or not fulfilling
Minor features present in the index patients are shown in Table 2. A significant difference in distribution was found between the internal and external collected data of the index patients \( [p = 0.02 \text{ (not fulfilling NF1 criteria)} \) and \( p = 4.4 \times 10^{-16} \text{ (fulfilling criteria)} \) which was mainly caused by the skewed percentage of observed patients having learning problems (both fulfilling and not fulfilling the NF1 criteria) and patients having speaking or behavior problems, epilepsy or short stature (only for those fulfilling the NF1 criteria). As expected, minor NF1 features in the overall group of index patients are more often observed in index patients fulfilling the NF1 criteria than in the subset of index patients not fulfilling the NF1 criteria (Table 2).

Genotype–phenotype correlations are shown in Table 3. NF1 patients with a microdeletion were found to have a higher incidence of special education compared to NF1 patients with an intragenic mutation in \( NF1 \) \( (p = 0.002, OR = 3.2, 95\% CI: 1.2–8.7) \). However, the Bonferroni-corrected significant level was only reached for the type 1 microdeletion group \( (p = 0.002, OR = 6.2, 95\% CI: 1.9–19.9) \). Similar results were obtained when excluding index patients not fulfilling the NIH NF1 criteria or when excluding intragenic single of multiple exon deletions/duplications. Patients with an \( NF1 \) missense mutation have a lower incidence of multiple neurofibromas and plexiform neurofibromas compared to NF1 patients with a different type of intragenic mutation in \( NF1 \) \( (p = 0.002, OR = 0.3, 95\% CI: 0.2–0.7 \) and \( p = 0.02, OR = 0.3, 95\% CI: 0.1–0.8) \). Both effects were only borderline significant (and not Bonferroni-corrected significant) when excluding index patients not fulfilling the NIH NF1 criteria \( (p = 0.05, OR = 0.5, 95\% CI: 0.2–1.0 \) and \( p = 0.05, OR = 0.3, 95\% CI: 0.1–1.0) \). No significant effect for sex and/or age was found in the regression analysis (data not shown). All patients having the c.2970_2972delAAT mutation \( (n = 7 \) with clinical data) were free of neurofibromas (for a more detailed phenotype, see http://www.lovd.nl/nf1).

In 28/1178 families with a pathogenic mutation \( (2.4\%) \), prenatal testing was requested once \( (n = 18) \) or a few times \( (n = 10) \). Almost all index patients of the families who requested prenatal analysis \( (93\%) \) fulfilled the NF1 criteria. In total, DNA of 46 fetuses was analyzed. An overview of the results of the prenatal analyses is given in Table S3 and Fig. S4. Prenatal testing was performed in case of a 50% risk of having an affected child \( (92.9\%) \) or in case of healthy tested parents with a previous child with confirmed NF1 \( (7.1\%) \). The majority of the fetuses was not affected \( (n = 29, 63\%) \), including both fetuses of the two healthy couples.

**Discussion**

During 18 years, almost 2000 index patients were tested for mutations in \( NF1 \) \( (> 800 \) with clinical data) in a diagnostic setting, i.e. to confirm the clinical diagnosis or to identify a pathogenic mutation for further analysis of family members, including prenatal testing. The
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Table 3. Genotype–phenotype correlations for NF1 (type 1) microdeletions vs NF1 intragenic mutations and NF1 missense mutations vs other types of NF1 intragenic mutations\(^a\)

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>NF1 intragenic mutation (n = 507)(^b)</th>
<th>NF1 microdeletion (n = 20)(^c)</th>
<th>P(^d)</th>
<th>NF1 microdeletion type 1 (n = 13)</th>
<th>P(^e)</th>
<th>NF1 missense mutation (n = 56)(^f)</th>
<th>P(^g)</th>
<th>Other types of NF1 intragenic mutation (n = 451)(^h)</th>
<th>P(^i)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major features</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple café au lait spots</td>
<td>452 (89.2)</td>
<td>18 (90.0)</td>
<td>NS</td>
<td>11 (84.6)</td>
<td>NS</td>
<td>52 (92.9)</td>
<td>NS</td>
<td>400 (88.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Freckling</td>
<td>343 (67.7)</td>
<td>15 (75.0)</td>
<td>NS</td>
<td>10 (76.9)</td>
<td>NS</td>
<td>32 (57.1)</td>
<td>NS</td>
<td>311 (69.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Multiple neurofibromas</td>
<td>220 (43.4)</td>
<td>12 (60.0)</td>
<td>NS</td>
<td>8 (61.5)</td>
<td>NS</td>
<td>14 (25.0)</td>
<td>NS</td>
<td>206 (45.7)</td>
<td>0.002(^j)</td>
</tr>
<tr>
<td>Plexiform neurofibroma</td>
<td>100 (19.7)</td>
<td>4 (20.0)</td>
<td>NS</td>
<td>3 (23.1)</td>
<td>NS</td>
<td>4 (7.1)</td>
<td>NS</td>
<td>96 (21.3)</td>
<td>0.02</td>
</tr>
<tr>
<td>Lisch nodules</td>
<td>173 (34.1)</td>
<td>6 (30.0)</td>
<td>NS</td>
<td>4 (30.8)</td>
<td>NS</td>
<td>13 (23.2)</td>
<td>NS</td>
<td>160 (35.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Optic glioma</td>
<td>30 (5.9)</td>
<td>2 (10.0)</td>
<td>NS</td>
<td>2 (15.4)</td>
<td>NS</td>
<td>4 (7.1)</td>
<td>NS</td>
<td>26 (5.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Bony dysplasia</td>
<td>27 (5.3)</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>2 (3.6)</td>
<td>NA</td>
<td>25 (5.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Positive family history NF1</td>
<td>46 (9.1)</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>5 (8.9)</td>
<td>NA</td>
<td>41 (9.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Minor features</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumors</td>
<td>29 (5.7)</td>
<td>2 (10.0)</td>
<td>NS</td>
<td>2 (15.4)</td>
<td>NS</td>
<td>6 (10.7)</td>
<td>NS</td>
<td>23 (5.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>59 (11.6)</td>
<td>3 (15.0)</td>
<td>NS</td>
<td>3 (23.1)</td>
<td>NS</td>
<td>6 (10.7)</td>
<td>NS</td>
<td>53 (11.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Macrocephaly</td>
<td>64 (12.6)</td>
<td>3 (15.0)</td>
<td>NS</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>7 (12.5)</td>
<td>NS</td>
<td>57 (12.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Short stature</td>
<td>23 (4.5)</td>
<td>1 (5.0)</td>
<td>NS</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>4 (7.1)</td>
<td>NS</td>
<td>19 (4.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Learning problems</td>
<td>146 (28.8)</td>
<td>9 (45.0)</td>
<td>NS</td>
<td>8 (61.5)</td>
<td>0.01</td>
<td>17 (30.4)</td>
<td>0.02</td>
<td>129 (28.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Special education</td>
<td>84 (16.6)</td>
<td>7 (35.0)</td>
<td>0.02</td>
<td>6 (46.2)</td>
<td>0.002(^j)</td>
<td>12 (21.4)</td>
<td>0.02</td>
<td>72 (16.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Mental retardation</td>
<td>25 (4.9)</td>
<td>2 (10.0)</td>
<td>NS</td>
<td>1 (7.7)</td>
<td>NS</td>
<td>3 (5.4)</td>
<td>NS</td>
<td>22 (4.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Speaking problems</td>
<td>81 (16.0)</td>
<td>3 (15.0)</td>
<td>NS</td>
<td>3 (23.1)</td>
<td>NS</td>
<td>11 (19.6)</td>
<td>NS</td>
<td>70 (15.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Behavior problems</td>
<td>58 (11.4)</td>
<td>4 (20.0)</td>
<td>NS</td>
<td>4 (30.8)</td>
<td>0.03</td>
<td>9 (16.1)</td>
<td>NS</td>
<td>49 (10.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>14 (2.8)</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>0 (0.0)</td>
<td>NA</td>
<td>2 (3.6)</td>
<td>NA</td>
<td>12 (2.7)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NA, not applicable; NS, not significant.
\(^a\)Only p < 0.05 are shown (in bold).
\(^b\)NF1 microdeletions: type 1 (n = 13), type 2 (n = 4), type 3 (n = 1) and atypical (n = 2).
\(^c\)p-Values resulting from the comparison of index patients with a pathogenic NF1 mutation with index patients having a NF1 microdeletion.
\(^d\)p-Values resulting from the comparison of index patients with a pathogenic NF1 mutation with index patients having a NF1 type 1 microdeletion.
\(^e\)p-Values resulting from the comparison of index patients with a pathogenic NF1 missense mutation with index patients having another type of NF1 intragenic mutation.
\(^f\)Significant after Bonferroni correction for multiple testing.

data collected in this period enabled us to give an overview of the genetic and clinical aspects of NF1 molecular diagnostics. The mutation detection rate in index patients fulfilling the NF1 criteria (80.9%) based on analysis of only genomic DNA is comparable with that presented by Griffiths et al. (78%) (21), higher than reported by Fahsold et al. (53%) (23) and slightly lower than indicated by Mattocks et al. (89%) (24) (Table S4). It is however unclear whether the mutations found in the cohort of Mattocks et al. are only clear pathogenic mutations (25). Higher detection rates are possible, but only when a multi-step mutation detection protocol is followed (e.g. including RNA analysis to detect ‘deep’ intronic mutations) (20, 26, 27). In our diagnostic laboratory mutation testing of ~250 genes, including NF1, is performed in a completely automated way, based on analysis of genomic DNA. We are aware of the fact that a small percentage of pathogenic mutations (‘deep’ intronic mutations, Alu insertions) will be missed using this approach. Misclassification of sequence variants is less likely to occur because we use an extensive in silico approach (see Appendix S1, use of Alamut) to identify the variants that are likely to have an effect on splicing. Referring physicians should be informed about the sensitivity of an NF1 analysis and we sometimes refer them to another laboratory offering NF1 analysis based on RNA, especially when the NF1 phenotype is unambiguous. Both referring physicians and molecular geneticists should also be aware that mosaic NF1 mutations can be missed in DNA isolated from blood. In these cases, DNA from tumor material can be helpful in identifying the pathogenic germline mutation. The exact percentage of mosaic NF1 mutations in our study population is unknown, because this entity was unfortunately not recorded in our database. It is also possible that index patients fulfilling the NF1 criteria and without any pathogenic mutation or UV in NF1 (n = 52, 9.8% in our study population) will not have NF1 at all. Their symptoms are overlapping...
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with other diseases/syndromes (e.g. Legius syndrome, 2/52 in our cohort) or symptoms are not observed or reported correctly. Finally, there is also a possibility of a mutation in another, yet unknown NF1 related, gene or regulatory element that also can result in NF1.

In the overall group of index patients, the mutation detection rate (59.3%) was lower compared to that in index patients fulfilling the NF1 criteria, but still comparable with literature (21), showing the importance of using the NIH NF1 criteria. We observed that these criteria are not always used (correctly) by the physician requesting an NF1 analysis. Therefore, physicians should be better informed (e.g. by establishing NF1 expertise centers) or the NIH NF1 criteria should be reviewed by the genetic laboratories prior before analysis, as is already done is some laboratories. The effect of an NF1 expertise center can be observed in our study population, as index patients with clinical data collected using our internal approach (see Methods section) fulfill the NF1 criteria more often than patients from outside our hospital (75.5% and 57.7%, respectively).

The majority of the index patients not fulfilling the NF1 criteria, but having an NF1 pathogenic mutation, is younger than 12 years (74%). This is not surprising as various NF1 symptoms appear age-specific and therefore (young) children do often not yet fulfill the NF1 criteria (7, 28–30). External referrals (mostly pediatricians) more often request NF1 testing for young children with, e.g. only CAL spots, as part of their differential diagnosis, than internal clinical geneticist. This also explains a part of the difference in percentage of index patients fulfilling the NF1 criteria between internal and external referrals.

Almost two third of the index patients with a pathogenic mutation is a sporadic case in our study population, which is considerably higher than then 50% previously reported in literature [20] and reviewed by Huson and Hughes (5). However, our high number of sporadic cases might be biased because the majority of the index patients was excluded because their sporadic/familial status was unknown. The percentage of sporadic cases drops to 27% when including this unknown group (63.3% of all index patients), making it difficult to determine the actual percentage of sporadic cases in our population. A positive family history was reported by the physicians in 11.9% of the index patients, which is in agreement with the number of familial cases (9.6%, including unknown group) found after genotyping.

The global distribution of mutation types varies between studies (also see Table S4). Our data are comparable to the study of Fahsold et al. who also used a DNA-based approach (23). The study of Griffiths et al., also using a DNA-based approach, showed a slightly higher percentage of NF1 microdeletions and missense mutations (21). These differences might be explained by the diversity of techniques used. The complete different distribution observed by a third group using a DNA-based approach is more difficult to explain (24). However, as reported before, they have misclassified some variants and their classification of pathogenic mutation, UV or polymorphism is sometimes unclear (25).

Our percentage of missense mutations is two times higher compared to studies using an more extensive approach (mRNA sequencing combined with other techniques) (20, 27). This difference can most likely be explained by the fact that some of our missense variants lead to splicing effects, which can only be observed using an RNA approach. Probably for the same reason our percentage of frameshift mutations is higher (some frameshift variants lead to splicing effects) and our percentage of splicing variants is lower compared to these two studies (also see Table S4).

We have identified 67 UVs in both patients fulfilling \((n = 49, 9.3%)\) and not fulfilling \((n = 18, 6.1%)\) the NF1 criteria. Although there are sometimes clues that these sequence variants are either neutral (e.g. silent mutations; 7.5% of the UVs) or pathogenic (e.g. splice site prediction algorithms indicate an effect of the variant on splicing), it is difficult to conclusively proof their status. In case an UV is identified, testing of the parents can result in classification of the sequence variant as being highly likely pathogenic in case the parents tested negative or likely benign in case the variant is present in one of the parents, and the parent has no NF1 symptoms at all. This indicates that clinical data can be essential in classification of UVs in NF1. Functional studies can also greatly help in elucidating the pathogenicity of genetic variants, however, these analyses are costly and time-consuming, especially to perform on a routine diagnostics basis. It would be very helpful to share NF1 variants in a global NF1 database, as was recently done for SPRED1 (22), to benefit from each others findings. Therefore, we have submitted all our sequence variants in a LOVD database (see Methods section) and we encourage other NF1 diagnostic laboratories to do the same.

The percentage of index patients fulfilling the NF1 criteria and having multiple CALs, freckling, multiple neurofibromas, or bony dysplasia is broadly similar to previous reports (6, 7, 30, 31). Plexiform neurofibromas, optic glioma, and Lisch nodules were found in, respectively, 21.8%, 6.8% and 38.4% of our index patients fulfilling the NF1 criteria, all a factor 2.3 lower than reported before (32–34). This 2.3-fold difference might be explained by an age effect. However, the percentages barely change when we exclude index patients <12 years. Minor NF1 features were, as expected, more often observed in index patients fulfilling the NF1 criteria. The only exception was the presence of tumors (several types, see Table S2; excluding those present in the major NF1 criteria), with frequencies similar in index patients fulfilling and not fulfilling the NF1 criteria. Most minor features were found (in index patients fulfilling the NF1 criteria) in a frequency comparable with that described in literature (30, 35–38). Speaking problems were observed in 16.7%, which is much lower than the 65% previously reported (39). This might be caused by the fact that mild speaking problems (e.g. poor articulation) might
be difficult to recognize by physicians not familiar with speech disorders. The minor NF1 feature ‘behavior problem’ is difficult to compare with literature because it is a collection of several different problems (e.g. activity disorder, aggression, and social interactions problems) (40).

Three NF1 patients in our study population also have juvenile myelomonocytic leukemia (JMML; see Table S2). One patient is compound heterozygous for a known NF1 pathogenic mutation (c.2033dupC, p.Ile679fs) and is mosaic for a NF1 microdeletion (most likely the somatic event, although no other tissues were available to test). The second patient is homozygous for a pathogenic intragenic mutation (c.6364G>A, p.Glu2122Lys; no NF1 copy number abnormality was detected with MLPA analysis), and the third patient has no NF1 mutation. The biallelic inactivation of NF1 in the first patients is because of constitutional inactivation of one allele and somatic inactivation of the other allele, whereas for the second patient mitotic recombination is the underlying mechanism (41–43). Homozygosity for a pathogenic NF1 mutation has been described in NF1-associated myeloid malignancies and gastrointestinal stromal tumors (42, 43). Because young children with a pathogenic NF1 mutation are at an increased risk of developing JMML (14% of the JMML cases have a clinical diagnosis of NF1) (44), we inform the referring physicians about the risk of JMML in case two pathogenic NF1 mutations are found in a patient <12 years.

A more severe clinical phenotype, including a higher prevalence of learning disabilities and dysmorphic features, has been reported for NF1 patients with an NF1 microdeletion compared to patients with an intragenic NF1 mutation (11–13). We could not confirm a higher prevalence of dysmorphic features in our study population. However, macrocephaly was the only dysmorphic feature recorded in our study. We did observe a significant higher prevalence of special education for NF1 patients with a microdeletion. The percentage of NF1 patients with a microdeletion having learning disabilities was also increased, however, not significantly. Apparently, in this group of patients, having a learning disability does not automatically mean that an individual also needs special education. We did not observe an evidently milder NF1 phenotype for patients with a missense mutation; only the presence of multiple neurofibromas was significantly reduced in this group of NF1 patients. All 14 index patients with a missense mutation and multiple neurofibromas fulfill the NIH NF1 criteria. Therefore, excluding the index patients not fulfilling the NIH NF1 criteria resulted in incidences for missense mutation (14/39 = 36%) and other intragenic mutations (199/372 = 53%) that differ not enough for this effect to remain significant. This clearly indicates that necessity of confirmation of this finding. Our testing for mutations was a DNA-based approach, and therefore, caution is needed when interpreting the results of the genotype–phenotype analysis for missense mutations vs other mutation types because the RNA consequences remain unknown for some of the missense mutations. Somatic mosaicism might explain some clinical variability of NF1. As the vast majority of our patients with a pathogenic mutation showed a clear heterozygous pattern for the mutation in DNA isolated from peripheral blood cells, low somatic mosaicism is excluded in these patients. Therefore, this variable could not be included in our regression model. The absence of multiple neurofibromas in our patients having the c.2970_2972delAAT mutation is in agreement with literature (14, 15).

Prenatal NF1 analysis was performed 46 times in our study population, indicating a need for prenatal NF1 testing, in agreement with results of a previous study (45). Even though the number of prenatal NF1 tests is relatively low, there is an increasing trend over the last few years till 2008. From 2009 onwards there has been a decrease in prenatal NF1 testing in the Netherlands that might be caused by the availability of preimplantation genetic diagnosis (PGD) as an alternative testing method (46, 47).

In conclusion, we have described our experiences from 18 years of NF1 molecular diagnostic testing. By analyzing the complete NF1 coding region and exon–intron boundaries, we were able to detect 1178 pathogenic mutations in 1985 index patients. As molecular techniques advance fairly rapidly over times, NF1 molecular testing might benefit from this in the future. For example, next-generation sequencing makes it possible to test several RASopathies genes or neurogenes at once. This technique might also be useful in finding new (‘major’) genes in patients with a clear phenotype and without a pathogenic mutation in known genes for that disease. Finally, confirmation of a clinical diagnosis, by identifying the pathogenic mutation, can result in a better treatment of patients and better counseling for their family members.

Supporting Information

The following Supporting information is available for this article:

**Fig. S1.** Different methods of testing neurofibromatosis type 1 in an 18 years period.

**Fig. S2.** Different types of pathogenic mutations present in index patients and without clinical data.

**Fig. S3.** (a) Histogram of the distribution of the unclassified variants over the NF1 exons. (b) Histogram of the distribution of the pathogenic mutations over the NF1 exons.

**Fig. S4.** Number of fetuses tested per year for a familial NF1 pathogenic mutation.

**Table S1.** Mutation detection rate in different subgroups.

**Table S2.** Different types of tumors observed in the index patients.

**Table S3.** Overview of the results of the prenatal analyses.

**Table S4.** Comparison of the distribution of mutation types, techniques and templates used, and mutation detection rate with literature.

**Appendix S1.** Using Alamut. Interpretation of sequence variants in Alamut.

Additional Supporting information may be found in the online version of this article.
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