Clinical and Genetic Findings in Children with Neurofibromatosis Type 1, Legius Syndrome, and Other Related Neurocutaneous Disorders

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Abstract: Pigmentary manifestations can represent an early clinical sign in children affected by Neurofibromatosis type 1 (NF1), Legius syndrome, and other neurocutaneous disorders. The differential molecular diagnosis of these pathologies is a challenge that can now be met by combining next generation sequencing of target genes with concurrent second-level tests, such as multiplex ligation-dependent probe amplification and RNA analysis. We clinically and genetically investigated 281 patients, almost all pediatric cases, presenting with either NF1 (n = 150), only pigmentary features (café au lait macules with or without freckling; (n = 95), or clinical suspicion of other RASopathies or neurocutaneous disorders (n = 36). The causative variant was identified in 239 out of the 281 patients analyzed (85.1%), while 42 patients remained undiagnosed (14.9%). The NF1 and SPRED1 genes were mutated in 73.3% and 2.8% of cases, respectively. The remaining 8.9% carried mutations in different genes associated with other disorders. We achieved a molecular diagnosis in 69.5% of cases with only pigmentary manifestations, allowing a more appropriate clinical management of these patients. Our findings, together with the increasing availability and sharing of clinical and genetic data, will help to identify further novel genotype–phenotype associations that may have a positive impact on patient follow-up.

Keywords: neurofibromatosis type 1; Legius syndrome; RASopathies; next generation sequencing; multiplex ligation-dependent probe amplification; RNA analysis

1. Introduction

Molecular diagnostic testing for Neurofibromatosis type 1 (NF1; MIM 162200) has improved considerably since identification of the first genotype–phenotype associations and an overlap disease,
Legius syndrome (LS; MIM 611431) [1]. Both disorders belong to the RASopathies [2–4], a group of autosomal dominant and phenotypically overlapping disorders caused by mutations in genes encoding for components of the Ras/mitogen activated protein kinase (MAPK) signaling pathway.

NF1 is a neurocutaneous condition characterized by multiple café au lait macules (CALMs), axillary and inguinal freckling, cutaneous neurofibromas, and iris Lisch nodules (LNs) [5,6]. Affected individuals show an increased susceptibility to developing benign tumors, such as plexiform neurofibroma, optic pathway glioma (OPG), and non-optic central nervous system glioma. Less common but potentially serious clinical manifestations are also reported [5–7].

With a birth incidence of approximately 1:3000, Neurofibromatosis type 1 is caused by dominantly inherited mutations in *NF1* (MIM 613113) [8], a complex gene [9] encoding for neurofibromin, a GTPase-activating protein that negatively regulates the Ras/MAPK signaling pathway [10]. Diagnosis of NF1 is still performed worldwide using clinical criteria formally codified in 1987 [6,11,12]. Nevertheless, clinical manifestations are variable and age related, with some distinguishing signs such as LNs and cutaneous neurofibromas appearing in late childhood or during puberty, further complicating clinical diagnosis of NF1 in young children, as well as in sporadic cases [6]. The differential diagnosis with other RASopathies is sometimes challenging due to the occurrence of signs of Noonan syndrome in NF1 patients (e.g., macrocephaly, Noonan-like facial features, short stature, and learning disabilities), as well as the presence of CALMs associated with some RASopathies (e.g., Noonan and LEOPARD syndromes) [13]. Milder NF1 phenotypes with pigmentary manifestations but without neurofibromas or OPG are associated with Met<sup>992</sup> deletion and Arg<sup>1809</sup> substitution in neurofibromin [14–16]. The 17q11.2 microdeletion fully encompassing *NF1* is known to be linked to a more severe phenotype with dysmorphic facial features, overgrowth or tall-for-age stature, significant delay in cognitive development, large hands and feet, hyper flexibility of joints, muscular hypotonia, and malignancies, such as the development of malignant peripheral nerve sheath tumor (MPNST) [17,18].

Initially described as an NF1-like phenotype, LS is caused by mutations in SPRED1 (MIM 609291) [1], which encodes Spred1, a member of the Sprouty/Spred protein family [19] and, similarly to neurofibromin, a negative regulator of the Ras/MAPK signaling pathway [20,21]. Legius syndrome is characterized by CALMs and freckling without neurofibromas or other typical NF1 features, such as LNs, bony lesions, and OPGs [1,22,23].

Milder or incomplete NF1 phenotypes observed in young-aged patients, sometimes related to specific mildly pathogenic *NF1* variants, clinically overlap with LS or, in some cases, with other RASopathies and constitutional mismatch repair deficiency (CMMRD; MIM 276300) [24], necessitating recourse to molecular testing. In this diagnostic scenario, the increasing use of next generation sequencing (NGS), and particularly customized targeted gene panels, provides the opportunity to investigate these clinically overlapping conditions in a time- and cost-saving manner. The concurrent use of second-level tests, such as multiplex ligation-dependent probe amplification (MLPA) or RNA analysis by RT-PCR and Sanger sequencing, can be useful to highlight specific classes of variants or to precisely characterize the effect of each variant on the protein product.

Here, we report our 10 year experience in molecular diagnosis of NF1 and LS, as well as other neurocutaneous conditions, in a large cohort of mostly pediatric patients. We also discuss how NGS and RNA analysis can improve the genetic characterization of patients, permitting differential diagnosis and guiding clinical follow-up.

2. Materials and Methods

2.1. Patient Recruitment and Clinical Classification

A total of 281 subjects, including 164 males (58.4%) and 117 females (41.6%), most of which were children (mean age 14 ± 12 years at the pre-test medical examination), were recruited for this study mainly from the Neurofibromatosis Referral Center at the University of Campania “Luigi Vanvitelli”
Department of Pediatrics. They were clinically evaluated according to the NIH diagnostic criteria and classified into six different groups.

Typical pigmentary manifestations (CALMs with or without freckling) were considered as the main clinical sign in children and were combined with distinctive NF1 features (LNs, OPG, bone dysplasia, and neurofibromas), age at the pre-test medical examination, and presence of affected first-degree relatives. Of the 281 subjects involved in this study, 150 received a definite clinical diagnosis of NF1 due to the presence of at least one NF1 distinctive sign and were further molecularly characterized only on the parents’ request or in presence of a milder phenotype \(n = 139\); Group 1), or when an NF1 microdeletion was suspected in the presence of a severe NF1 phenotype \(n = 11\); Group 2).

An age-based categorization was established mainly to prioritize NF1/SPRED1 mutation analysis, relying on the fact that some typical NF1 features, such as LNs and neurofibromas, may not be yet present in children aged <10 years. A further 44 patients with apparently pigmentary manifestations only, without affected first-degree relatives, and aged \(\leq 9\) years were prioritized for mutation analysis of NF1 and, subsequently, SPRED1 (Group 3), while 51 patients either with pigmentary manifestations only, without affected first-degree relatives, and aged \(\geq 10\) years \(n = 31\); Group 4), or with at least one affected first-degree relative \(n = 20\); Group 5) were prioritized for mutation analysis of SPRED1 and subsequently NF1. Finally, 36 patients with clinical features suggestive of a RASopathy or other neurocutaneous disorders formed Group 6.

Samples were also collected from patients’ affected or unaffected relatives \(n = 167\) when necessary.

Written informed consent for DNA analysis was obtained from all the subjects investigated or from their legal guardians at the pre-test medical examination, including explicit consent for future use of data for research purposes, according to the Declaration of Helsinki. Approval for the study was obtained from the Ethics Committee of the University of Campania “Luigi Vanvitelli” \(#254-05/02/2019\).

For each subject, blood samples were collected in PAXgene Blood RNA Tubes (Qiagen, Hilden, Germany) or Tempus Blood RNA Tubes (Life Technologies, Carlsbad, CA, USA) to prevent illegitimate splicing during subsequent RNA extraction and analysis \[25,26\]. Genomic DNA was also extracted using standard procedures.

2.2. Primer Design for NF1 and SPRED1 Mutation Screening

NF1 pseudogenes occur on different human chromosomes \[27\]. To minimize the amplification of targets other than the expected templates, we used the Primer-BLAST tool \[http://www.ncbi.nlm.nih.gov/tools/primer-blast/\] for primer design.

For RNA analysis of the entire coding sequences of NF1 and SPRED1 (RefSeq: NM_000267.3 and NM_152594.3, respectively), we designed primer pairs that amplified partially overlapping fragments of 500–700 bp (Supplementary Materials Table S1). For both genes, genomic oligonucleotide pairs were also designed to amplify each exon and its intronic flanking regions (Supplementary Materials Table S2).

2.3. Mutation Screening by RT-PCR

For a large number of subjects investigated, NF1 and SPRED1 were analyzed at the cDNA level. Total RNA was extracted using PAXgene Blood RNA Kit (Qiagen, Hilden, Germany) or Tempus Spin RNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturers’ specifications. RNAs were then retro-transcribed using SuperScript III RT (Invitrogen, Carlsbad, CA, USA) and random primers, according to the manufacturer’s instructions. Single-strand cDNAs were used in later experiments.

The RT-PCR was performed in a final volume of 20 µL containing 2 µL cDNA, 1X PCR Buffer II (Applied Biosystems, Foster City, CA, USA), 1 mM MgCl₂, 1 mM dNTPs, 0.5 µM of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Cycling conditions consisted of a first step at 96 °C for 7 min followed by 30 cycles of 30 s at 96 °C, 1 min at 63 °C, and 3 min plus 3 s/cycle at 68 °C.
The RT-PCR products were first analyzed by agarose gel electrophoresis to highlight possible unexpected products. For each sample, overlapping fragments covering the entire \textit{NF1} or \textit{SPRED1} coding sequence were subsequently analyzed by bidirectional sequencing.

\textbf{2.4. Targeted NGS-Based Mutational Screening}

To extend mutation analysis to other genes involved in RASopathies, neurocutaneous disorders, and other genetically determined conditions with pigmented manifestations in pediatric age, we designed a customized target NGS panel using HaloPlex technology (Agilent Technologies, Santa Clara, CA, USA). We selected 35 known disease-causing genes (Supplementary Table S3). The custom panel design also included genes identified as potential interactors of these 35 disease genes using two different bioinformatic tools, STRING and GeneMania [28,29]. Only genes matching both tools were added to the design.

Enrichment of target sequences of all selected coding genes was performed using the HaloPlex Target Enrichment System for Illumina (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. For each sample, 200 ng of genomic DNA was digested with eight different restriction enzymes to create the fragment library and hybridized for 16 h to specific probes for Illumina sequencing. After capture of the biotinylated target DNA using streptavidin beads, nicks in the circularized fragments were closed by a ligase. Finally, the captured target DNA was eluted by NaOH and amplified by PCR. The amplified target molecules were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics, Chaska, MN, USA). The enriched target DNA in each library sample was validated and quantified by microfluidic analysis using the Bioanalyzer High Sensitivity DNA Assay Kit and 2100 Bioanalyzer Expert Software (Agilent Technologies). Samples were run on a NextSeq500 System (Illumina, San Diego, CA, USA), generating 150 bp-long paired-end reads.

Generated sequences were analyzed using an in-house pipeline designed to automate the analysis workflow [30]. Average coverage for all the experiments was 70\(\times\) and at least 20\(\times\) for 98% of the target. Paired sequencing reads were aligned to the reference genome (UCSC, hg19 build) using a Burrows–Wheeler Aligner, and sorted with SAMtools and Picard (http://picard.sourceforge.net). Calling of single nucleotide variants (SNVs) and small insertions/deletions (Ins/Del) was performed with the Genome Analysis Toolkit (GATK) [31] with parameters adapted to HaloPlex-generated sequences. The called SNVs and Ins/Del variants were annotated using ANNOVAR [32], reporting variant position in RefSeq [33], amino acid change, presence in dbSNP v151 [34], frequency in the NHLBI Exome Variant Server (http://evs.gs.washington.edu/EVS), 1000 genomes [35], and Exome Aggregation Consortium (ExAC) browser (http://exac.broadinstitute.org) projects, multiple cross-species conservation [36], and prediction scores of damaging on protein activity [37].

\textbf{2.5. Multiplex Ligation-Dependent Probe Amplification}

To identify complete or partial deletions/duplications in \textit{NF1}, \textit{SPRED1}, \textit{NF2}, \textit{TSC1}, and \textit{TSC2} genes, MLPA assays were performed using SALSA MLPA P081/P082 NF1 kit, SALSA MLPA P295 SPRED1 kit, SALSA MLPA P044 NF2 kit, SALSA MLPA P124 TSC1 kit, and SALSA MLPA P337 TSC2 kit, respectively (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer’s recommendations. When \textit{NF1} microdeletions were detected, SALSA MLPA P122 NF1 kit (MRC-Holland) was also used to better define breakpoint boundaries.

Briefly, denatured genomic DNA (100 ng) was added to the MLPA mix and the probes were allowed to anneal overnight before the subsequent ligation reaction was performed. Polymerase chain reaction (PCR) was carried out with 6-carboxyfluorescein (FAM)-labeled primers using 5 \(\mu\)L of the ligation reaction as the template. The PCR products were then separated on an ABI 3130XL automatic DNA sequencer (Life Technologies), including at least three normal DNA samples in each batch of the MLPA assays for the subsequent normalization of results.

The MLPA data analysis was performed using the Coffalyser.Net package (MRC-Holland). Relative amounts of probe-amplified products were compared with reference samples to determine
the copy number of target sequences. Values under a threshold of 0.7 and over a threshold of 1.3 for multiple adjacent probes indicate the presence of a deletion or duplication, respectively.

2.6. Real-Time PCR

To confirm copy-number mutations identified by MLPA, quantitative amplification of the specific genomic regions was performed on CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using iQ SYBR Green Supermix (Bio-Rad Laboratories) according to the manufacturer’s instructions. Uracil N-glycosylase (Amperase UNG, Life Technologies) was used to prevent PCR carry-over contamination. Each assay was performed in triplicate and the results were normalized and analyzed using CFX Manager software version 1.5 (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Validation of Variants by Sanger Sequencing

The PCR products were double-strand sequenced using BigDye Terminator sequencing chemistry (Life Technologies) and analyzed on an ABI 3130xL automatic DNA sequencer (Life Technologies, Carlsbad, CA, USA). Automatic variation calling was obtained by analyzing sequencing data (ABI file) using Mutation Surveyor software version 3.24 (SoftGenetics, State College, PA, USA), followed by careful inspection of the electropherograms to minimize variant loss.

3. Results

Over the last decade, approximately 600 patients suspected of being affected by NF1 or an NF1-like condition, or by RASopathies and other neurocutaneous disorders, were clinically evaluated at our Neurofibromatosis Referral Center in accordance with the NIH diagnostic criteria. For these patients, genetic testing was proposed whenever it may have been useful to confirm the clinical diagnosis. A total of 281 probands gave their informed consent and were included in this study, and molecular analysis was extended to their affected or unaffected relatives \( n = 167 \) when necessary. To optimize the use of genetic testing in discriminating NF1 versus LS and other neurocutaneous disorders in childhood, patients were classified into six groups (see Materials and Methods) and prioritized according to their clinical features.

3.1. Molecular Diagnosis

Results of molecular diagnosis for each patient group are summarized in Table 1. The causative variant was detected in 239 out of 281 patients analyzed (85.1%), with only 42 undiagnosed patients (14.9%). Both \( NF1 \) and \( SPRED1 \) were mutated in 73.3% and 2.8% of cases, respectively. The remaining 8.9% presented causative variants in different genes.
Table 1. Results of the molecular diagnosis by patient group.

| Group                                | Criteria for Molecular Testing                                                                 | Number of Selected Patients | Number of Mutated Patients | Mutation Detection Rate (%) | Number of Patients without Molecular Diagnosis (%) |
|--------------------------------------|------------------------------------------------------------------------------------------------|----------------------------|----------------------------|----------------------------|---------------------------------------------------|
| 1                                    | Clinical diagnosis of NF1 (test requested by parents or milder phenotype)                     | 139                        | 136 (NF1 = 136, SPRED1 = 0, OTHER = 0) | 97.8% (NF1 = 97.8%, SPRED1 = 0.0%, OTHER = 0.0%) | 3 (2.2%)                                          |
| 2                                    | Severe NF1 phenotype with suspicion of 17q11.2 microdeletion                                   | 11                         | 11 (NF1 = 11, SPRED1 = 0, OTHER = 0) | 100.0% (NF1 = 100.0%, SPRED1 = 0.0%, OTHER = 0.0%) | 0 (0.0%)                                          |
| 3                                    | Isolated CALMs in patients without affected first-degree relatives and age ≤ 9 y              | 44                         | 29 (NF1 = 28, SPRED1 = 1, OTHER = 0) | 65.9% (NF1 = 63.6%, SPRED1 = 2.3%, OTHER = 0.0%) | 15 (34.1%)                                        |
| 4                                    | Isolated CALMs in patients without affected first-degree relatives and age ≥ 10 y            | 31                         | 20 (NF1 = 19, SPRED1 = 1, OTHER = 0) | 64.5% (NF1 = 61.3%, SPRED1 = 3.2%, OTHER = 0.0%) | 11 (35.5%)                                        |
| 5                                    | Isolated CALMs in patients and at least one affected first-degree relative                   | 20                         | 17 (NF1 = 11, SPRED1 = 6, OTHER = 0) | 85.0% (NF1 = 55.0%, SPRED1 = 30.0%, OTHER = 0.0%) | 3 (15.0%)                                         |
| 6                                    | Other RASopathies or neurocutaneous disorders                                                | 36                         | 26 (NF1 = 1, SPRED1 = 0, OTHER = 25) | 72.2% (NF1 = 2.7%, SPRED1 = 0.0%, OTHER = 69.4%) | 10 (27.8%)                                        |
| Total                                |                                                                                              | 281                        | 239 (NF1 = 206, SPRED1 = 8, OTHER = 25) | 85.0% (NF1 = 73.3%, SPRED1 = 2.8%, OTHER = 8.9%) | 42 (15.0%)                                        |

Notes: NF1 = neurofibromatosis type 1; CALMs = café au lait macules; OTHER = other disease genes investigated (the identified causative variants are reported in Table A4).
In subjects with a clinical diagnosis of NF1 (Groups 1, 2), the mutation detection rate was 98% (147/150), similarly to previously reported findings [38]. When pigmentary manifestations were the only early clinical sign (Groups 3–5), the mutation detection rate fell to 69.5% (66/95), with SPRED1 accounting for around 8.4% (8/95) of identified causative variants. Interestingly, the lowest detection rate (64.5%) was obtained for subjects presenting only CALMs, aged ≥ 10 years at the pre-test medical examination, and without affected first-degree relatives (Group 4). In contrast, subjects presenting only CALMs and with at least one affected first-degree relative (Group 5) achieved a higher mutation detection rate (85%), with NF1 and SPRED1 accounting for 55% and 30%, respectively.

In subjects with a clinical suspicion of a RASopathy or neurocutaneous disorder, the mutation detection rate was 72.2% (26/36), with variants distributed in ten different genes, mainly PTPN11 (22.2%), and with only one causative variant in NF1 (2.7%).

3.2. NF1 Mutation Screening

By combining NGS, direct sequencing of RT-PCR products, and MLPA analysis, we identified 169 different causative variants along NF1 (Table A1). As expected, 33.1% of these (56/169) were novel variants. Single-nucleotide substitutions and single or very short deletion/insertion of bases accounted for 67.4% (114/169) and 30.2% (51/169) of the identified causative variants, respectively. Wider deletions or duplications at NF1 locus made up the remaining 2.4%. Excluding this last class of mutations, variants were distributed in almost all NF1 exons (Supplementary Materials Figure S1) and only 21 were recurrent variants, being present in at least two unrelated patients.

Base substitutions resulted in 31 nonsense variants (27.2%), three of which were novel, 34 missense variants (29.8%), eight of which were not previously reported, and 42 variants differently affecting splicing (36.8%), mainly resulting in a frameshift of NF1 coding sequence.

Novel missense variants were further investigated, considering segregation in familial cases or their de novo occurrence. In support of their pathogenic effect, these amino acid changes were predicted to be deleterious by common in silico prediction programs (SIFT, Polyphen-2, and Mutation Taster; Supplementary Materials Table S4) [39–41], and were not annotated in the gnomAD and ExAC browsers [42].

Variants affecting mRNA splicing are common in NF1 [25,38]. In our study, they represent 25.4% (43/169) of all identified causative variants, with 34 variants differently perturbing canonical splice acceptor or donor sites, five variants within exons creating de novo splice sites and resulting in the loss of a part of the exon, and four deep intronic mutations activating cryptic splice sites (Supplementary Materials Table S5). This last type would likely be underestimated without RNA analysis. In our cohort of NF1 patients, this class of mutation accounted for 2.4% of all identified variants (4/169), three of which were not previously reported.

Complete NF1 microdeletion and other rearrangements partially involving NF1 were detected by MLPA analysis. In line with other reports [43,44], NF1 microdeletions at 17q11.2 were present in 4.9% (10/205) of patients with an identified causative variant in NF1, while two intragenic deletions of exons 15(11)–36(27b) and exons 28(22)–29(23) and a duplication of exons 37(28)–51(42) in NF1 were identified in three further patients.

3.3. SPRED1 Mutation Screening

We identified eight different causative variants in SPRED1 (Table A2), three of which were novel. Single nucleotide substitutions resulted in four already reported nonsense variants and one missense variant. We also identified a novel 5 bp deletion in a large family with 10 affected individuals and a novel one-base duplication in another family. The MLPA analysis characterized a sporadic case with an intragenic deletion of the last two exons and the 3’UTR of SPRED1.
3.4. Phenotype-Genotype Overview

Table A3 summarizes the clinical features of 245 probands suspected of being affected by NF1 or an NF1-like condition evaluated at the pre-test medical examination (T0) and after genetic testing (T1). Based on the NIH diagnostic criteria, a clinical diagnosis of NF1 was achieved in 150 patients (Groups 1 and 2), 99.3% of which presented CALMs either with (78.7%) or without (18%) freckling associated with LNs (55.4%), OPG (14%), bone dysplasia (2%), cutaneous or plexiform neurofibromas (62.7%), or an affected first-degree relative (41.3%). One case (Family ID 108) did not quite meet the NIH diagnostic criteria but was nevertheless included in Group 1 due to the presence of neurofibromas at age 5 months, and minor clinical features such as macrocephaly, nevus anemicus, psychomotor delay, and thorax abnormalities. About 98% of these clinically diagnosed patients presented a causative NF1 variant that resulted in truncated or absent neurofibromin (75.5%), or in in-frame deletions (10.9%) or single substitutions (13.6%) of amino acids.

The remaining 95 patients (Groups 3–5) only presented CALMs (100%), with (29.5%) or without (70.5%) freckling and were negative for the other NIH diagnostic criteria at the pre-test medical examination. Among these, 75 patients were sporadic cases aged ≤ 9 years (n = 44; Group 3) or ≥10 years (n = 31; Group 4), while the remaining 20 patients were familial cases (Group 5). In Group 3, a causative variant in NF1 was identified in 63.6% of patients, resulting in truncated or absent neurofibromin (67.9%), or in in-frame deletions (10.7%) or single substitutions (21.4%) of amino acids. In Group 4, which presented the lowest mutation detection rate (61.3%), NF1 was still the most commonly involved gene with an increased percentage of variants causing in-frame deletions or single substitutions of amino acids (61.1%) compared to those resulting in truncated or absent neurofibromin (38.9%). Only one causative variant was detected in SPRED1 (Family ID 157). In Groups 3 and 4, typical NF1 clinical features subsequently appeared in only 11 patients with a causative variant in NF1 identified by genetic testing (T1). For Groups 3 and 4, 48% of patients (36/75) presented only CALMs without any other typical NF1 feature, even after genetic testing (T1), thus not falling within the NIH diagnostic criteria. Interestingly, a causative variant in NF1 was identified in 30.6% of cases (11/36). In only one case (Family ID 224) was a causative variant in SPRED1 detected. Finally, NF1 and SPRED1 were similarly mutated in patients from Group 5. Variants in NF1 (55%) gave rise to truncated or absent neurofibromin in only two patients, while SPRED1 variants (30%) mainly caused haploinsufficiency. Again, in Group 5, no further typical NF1 clinical features subsequently appeared in patients with an NF1 variant identified by genetic testing (T1).

Neurofibromatosis bright objects were the most frequently observed of all minor clinical features (Table A3), presenting in 20.8% of cases. Learning disabilities and/or speech problems were found in 18.8% of patients, while thorax abnormalities, macrocephaly, leg length discrepancy, and scoliosis in 16.7%. Noonan-like facial features (7.8%), intellectual disability (6.9%), and behavior problems (5.7%) were also observed. Less common but potentially serious malignancies, including malignant peripheral nerve sheath tumor (MPNST), leukemia, and rhabdomyosarcoma, accounted for 2.9% of cases, while vascular alterations such as Moyamoya syndrome and pulmonary stenosis were observed in 2% and 1.6% of cases, respectively.

3.5. Mutation Screening in Non-NF1 or NF1-Like Conditions and Unsolved Cases

By combining NGS and MLPA analysis, we also investigated 36 patients with clinical features suggestive of a RASopathy or neurocutaneous disorder (Group 6; Table A4). Among these, 14 patients with RASopathy features presented causative variants in PTPN11 (8/14), SOS1 (2/14), PPP1CB (1/14), and NF1 (1/14), 14 patients diagnosed with tuberous sclerosis complex (TSC) showed variants in TSC1 (3/14) and TSC2 (5/14), five patients with Neurofibromatosis type 2 or Schwannomatosis presented causative variants in NF2 (3/5) and LZTR1 (1/5), while a variant in PTEN and KIT was identified in two other cases with a clinical diagnosis of Cowden syndrome and Piebaldism. Six of the identified causative variants were not previously reported (Table A4 and Supplementary Materials Table S4). Biallelic germline variants in mismatch repair (MMR) genes are known to be responsible for CMMRD.
Although this condition is associated with a broad spectrum of early-onset tumors often associated with NF1 features, especially CALMs [24,45], no causative variants in MMR genes were found in our cohort.

In 42 unsolved cases, we also investigated for variants in candidate genes, considering different models of inheritance. For one patient only, we identified a rare missense heterozygous variant in MAPK3 (NM_001109891.1:c.601C>A; p.Leu201Met) not present in our internal database or in any public databases. Although MAPK3 encodes for a member of the MAP kinase family [46], any pathogenic role for the observed variant is currently only speculative.

4. Discussion

In recent years, NGS has greatly improved the molecular diagnosis of inherited diseases, particularly in the case of genetically heterogeneous and clinically overlapping conditions. Our experience further supports the diagnostic value of NGS and shows how a targeted NGS-based entry-level test [47–49] combined with RNA and MLPA analysis for a complete molecular characterization [50,51] achieves a high mutation detection rate and is extremely useful in addressing differential diagnosis of NF1 and overlap diseases. In fact, we obtained a molecular diagnosis in about 85% of cases investigated.

For patients with a clinical diagnosis of NF1 (Groups 1, 2), 98% carried an NF1 causative variant, resulting in truncated or absent neurofibromin in 75.5% of cases. All subjects presented CALMs, with freckling in 86% of cases, as well as the most common typical NF1 features including neurofibromas (64.7%), LNs (59.4%), and OPG (16%). Recently, causative variants in the cysteine-serine-rich domain (CSRD; residues 543–909) were positively associated with OPG [52]. Among the 24 NF1 patients presenting OPG investigated, seven showed an NF1 variant within the CSRD domain. Intellectual and/or learning disability or speech problems were present in about 26.7% of cases, while 8.7% showed Noonan-like facial features [53]. Malignancies and vascular alterations, such as Moyamoya syndrome, were observed in 4% and 4.7% of cases, respectively [54]. A large NF1 family with co-occurrence of Moyamoya syndrome in two first cousins (Family ID 16) was recently further investigated by whole exome sequencing, which identified MRVI1 as a susceptibility gene for Moyamoya syndrome in NF1 [55]. Of 11 patients with a more severe NF1 phenotype (Group 2) suggestive of NF1 microdeletion [17,18,56], a 17q11.2 microdeletion was in fact detected in six cases. Among these, two patients later died from MPNST, frequently seen in NF1 microdeletion patients [18,56]. Truncating variants were present in four other cases with severe NF1 phenotype, and removed large part of the protein sequence with its functional domains. The remaining patient (Family ID 119) showed an in-frame deletion (p.Tyr1614_Tyr1618del) falling in the Sec14-like domain of neurofibromin. This patient showed moderate intellectual disability with learning difficulties and speech problems, macrocephaly, dysmorphic facial features, tall stature, and skeletal anomalies (leg length discrepancy, dystrophic scoliosis, and vertebral scalloping), a small number of subcutaneous neurofibromas, and medullary unidentified bright objects [57]. Interestingly, the Sec14-like domain of neurofibromin interacts with valosin-containing protein (VCP), regulating dendritic spine density [58]. Dominantly inherited VCP mutations cause inclusion body myopathy with Paget disease of bone and frontotemporal dementia [59].

Molecular investigation was critical in achieving a clinical diagnosis for patients with only pigmentary features (CALMs with or without freckling; Groups 3–5); for these patients we were able to detect the causative variant in 69.5% of cases. This result highlights the clinical utility of genetic testing, particularly in pediatric age, even in those cases which do not fall within the NIH diagnostic criteria, driving the patient’s clinical early follow-up and management. In sporadic cases (n = 75; Groups 3, 4), NF1 was mutated in 62.7% of patients, with only two (Family ID 157 and 224) presenting a variant in SPRED1 causing haploinsufficiency. Among the NF1 variants, 55.3% resulted in haploinsufficiency, while the rest were in-frame deletions or single substitutions of amino acids. Subsequent to genetic testing, additional typical NF1 features (LNs and/or neurofibromas) appeared in 11 NF1 patients, 10 of which had a truncating variant in NF1. The remaining NF1 patients presented a mild phenotype,
with only CALMs either with or without freckling, in some cases complicated by speech and learning problems, short stature, and macrocephaly. Of these, three cases carried the Arg_{1809} substitution in neurofibromin [15,16].

The causative variant was detected in 85% of patients with pigmentary manifestations also present in at least one affected first-degree relative (n = 20; Group 5), with NF1 and SPRED1 similarly involved. Missense variants were the most common type of variants found in NF1, with the Arg_{1809} substitution accounting for 45.5% [15,16]. In Group 5, we also molecularly diagnosed the highest percentage of LS (30%), which appears to be primarily associated with inherited mutant alleles, unlike NF1, in which de novo variants frequently occur.

Noonan-like features can be observed in NF1 patients. A neurofibromatosis-Noonan Syndrome (MIM 601321) was reported [60,61] and linked to variants in NF1 [53], but also to the co-occurrence of independent variants in NF1 and PTPN11 in the same patient [62,63]. The NGS analysis excluded additive variants in Noonan syndrome-causing genes in our molecularly diagnosed NF1 patients with combined NF1 and Noonan-like features.

We also extended our investigation to 36 patients with clinical features suggestive of other RASopathies or neurocutaneous disorders (Group 6), identifying a causative variant in line with clinical suspicion in 25 cases (Table A4). In one child (Family ID 284) referred by endocrinologists due to clinical suspicion of Noonan syndrome, we did not find any causative variants in Noonan syndrome-causing genes but, unexpectedly, an unreported and maternally inherited missense variant in NF1 (p.Glu1198Lys). At genetic counseling, the patient presented CALMs, Noonan-like facial features and habitus (short stature, relative macrocephaly, hypertelorism, thoracic asymmetry, and sternum carinatum). Her mother also presented a very small number of CALMs, mild Noonan-like facial features, soft hands and feet, and short stature. Further investigation of this mild phenotype in other patients with the same NF1 variant may help characterize a possible genotype-phenotype association. This case is illustrative of the diagnostic overlap between these two conditions, as well as the recently reported case [64] of a child (Family ID 187) carrying an SOS1 variant inherited from his mother, who initially received a diagnosis of NF1 due to the spinal nerve enlargement resembling neurofibromas.

The combined or alternative use of NGS and RNA analysis was able to precisely characterize the functional effect of each causative variant identified in NF1 and SPRED1, in some cases overcoming the limits of each approach when performed individually. Specifically, some missense or nonsense variants in NF1 caused exon skipping or generated cryptic splice sites. Moreover, similarly exon-skipped NF1 transcripts were caused by different genomic variants proximal to the same splice site. For some of these skipped exons, including 15(11), 37(27a), and 46(37), phenotype variability was observed in affected patients. Deep intronic mutations activating cryptic splice sites were only detectable by RNA analysis and accounted for 2.4% of all the causative variants we identified. Conversely, three variants in the first exon of NF1 causing RNA decay were only detectable by NGS analysis. Considering recently reported (and potential novel) genotype-phenotype correlations [15,16,18,52,65–68], evaluating the functional effect of genomic variants can have a positive impact on patients’ clinical follow-up.

Of the unsolved cases, only four patients had a definite clinical diagnosis of NF1 due to the presence of typical pigmentary manifestations combined with at least one additional distinctive NF1 clinical feature. One of these (Family ID 114) was subsequently diagnosed as having a mosaic form of NF1 because of the peculiar distribution of CALMs restricted to the right side of the trunk and LNs only in the right eye. As suggested by the lowest detection rate, isolated CALMs (Groups 3–5) are associated with a lower possibility of obtaining a molecular diagnosis. This might be due to mosaicism or to the existence of other genetic causes of isolated pigmentary manifestations yet to be discovered. In Group 6, six unsolved cases had a clinical diagnosis of TSC. However, about 15%–20% of TSC patients may have unidentifiable mutations or a mosaicism [69,70]. Finally, one patient (Family ID 219) with a clinical diagnosis of Schwannomatosis and negative for mutations in NF2, LZTR1, and SMARCB1 is under investigation for a somatic mosaicism in NF2.
5. Conclusions

Our findings highlight the clinical and diagnostic challenges of a pediatric referral center for neurocutaneous disorders, demonstrating how a combined NGS-based approach can assist clinicians in the diagnosis of NF1 as well as other neurocutaneous disorders and overlapping conditions. We categorized patients based on clinical signs and considered an NF1 diagnosis certain only when other distinctive signs besides CALMs and freckling were present, achieving a very high detection rate and providing a precise characterization of identified causative variants. Our results also highlight how it can still make sense to prioritize patients for NF1 mutation analysis when presenting only CALMs, typical of NF1 in term of number and diameter, and independently from the age at clinical observation. Our categorization suggests that older patients showing only CALMs tend to remain without a definite molecular diagnosis. The RNA analysis facilitates in interpreting the functional effect of genomic variants and can drive the identification of new genotype-phenotype correlations, potentially impacting on the clinical management of NF1 pediatric patients. Through the sharing of clinical and molecular data among members of the scientific community, we are confident it will be possible to identify novel genotype–phenotype correlations and ultimately improve patient outcomes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/10/8/580/s1, Figure S1: Distribution of identified variants in exons of NF1, Table S1: List of primer pairs designed to amplify overlapping fragments for RNA analysis of the entire coding sequences of NF1 and SPRED1, Table S2: List of genomic primer pairs designed to amplify exons and intronic flanking regions of NF1 and SPRED1, Table S3: List of genes included in the customized target NGS panel, Table S4: In silico prediction of deleterious effects and segregation analysis for unreported missense variants, Table S5: In silico prediction of splice score for deep intronic mutations in NF1 (www.fruitfly.org/seq_tools/splice.html).

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Conflicts of Interest: The authors declare no conflict of interest.
## Appendix A

**Table A1.** Unique variants identified in NF1 (RefSeq NM_000267.3).

| Family ID | Group | Exon | Type | Genomic | cDNA | Effect | Protein | ClinVar/HGMD/LOVD ID |
|-----------|-------|------|------|---------|------|--------|---------|---------------------|
| 216 (3)   | 1     | 01   | SNV  | 3G>A    | RNA decay | RNA decay | ? | LOVD: NF1_001130 |
| 220 (1)   | 1     | 01   | SNV  | 59A>C   | RNA decay | RNA decay | ? | New |
| 212 (1)   | 1     | 01   | SNV  | 60G>C   | RNA decay | RNA decay | ? | New |
| 35 (2)    | 1     | 02   | SNV  | 128T>C  | Missense | Leu437Pro | LOVD: NF1_000049 |
| 67 (1)    | 1     | 03   | SNV  | 277T>C  | Missense | Cys93Arg  | LOVD: NF1_002264 |
| 294 (1)   | 3     | 03   | SNV  | 288+1delG | Splicing | Gln97Asn*6 | LOVD: NF1_001615 |
| 81 (1)    | 3     | 03   | SNV  | 288+1G>A (Splicing) | Splicing | Arg69_Gly96del | LOVD: NF1_001484 |
| 262 (1)   | 5     | 03   | SNV  | 288+4A>G | Splicing | Arg69_Gly96del | LOVD: NF1_000270 |

**Intronic cryptic splice site**

| Family ID | Group | Exon | Type | Genomic | cDNA | Effect | Protein | ClinVar/HGMD/LOVD ID |
|-----------|-------|------|------|---------|------|--------|---------|---------------------|
| 93 (3)    | 1     | 03   | DIM  | 289-2956C>T (cryptic splice site) | RNA decay | RNA decay | ? | New |
| 72 (1)    | 1     | 04   | DEL  | 363del | Frame-shift | Hist122Thrfs*43 | New |
| 31 (1)    | 1     | 05   | DEL  | 499_502del | Frame-shift | Cys167Glufs*10 | LOVD: NF1_000605 |
| 51 (1)    | 1     | 05   | SNV  | 574C>T  | Nonsense | Arg192* | LOVD: NF1_000702 |
| 121 (1)   | 3     | 07   | SNV  | 677T>A  | Missense | Thr223Arg | LOVD: NF1_000800 |
| 128 (1)   | 3     | 08   | SNV  | 818T>C  | Missense | Leu273Pro | New |
| 221 (1)   | 1     | 09   | DEL  | 1019_1020del | Frame-shift | Ser340Cysfs*12 | LOVD: NF1_000005 |
| 172 (1)   | 1     | 10   | DEL  | 1110del | Frame-shift | Ala371Gln*5 | New |
| 49 (1)    | 3     | 10   | DEL  | 1123del | Frame-shift | Leu375* | New |
| 296 (1)   | 4     | 10   | SNV  | 1144C>T | Missense | Ser382Pro | New |
| 46 (1)    | 1     | 10   | SNV  | 1185+1G>A (splicing) | Splicing | Asn355_Lys395del | LOVD: NF1_000019 |
| 218 (1)   | 1     | 10   | SNV  | 1185G>T (splicing) | Splicing | Asn355_Lys395del | LOVD: NF1_000019 |
| 125 (1)   | 3     | 11   | SNV  | 1186-3G (splicing) | Splicing | Ile396Glu*17 | New |
| 292 (1)   | 1     | 11   | SNV  | 1198C>T | Non-sense | Gln457* | LOVD: NF1_000024 |
| 110 (1)   | 3     | 11   | INS  | 1249dup | Frame-shift | His415Profs*14 | ClinVar: 426651 |
| 23 (2)    |       |      |      |         | Nonsense | Arg416* | LOVD: NF1_000034 |
| 152 (1)   | 1     | 11   | SNV  | 1246C>T | Splicing | Asn355_Lys395del | LOVD: NF1_000019 |
| 45 (1)    | 3     | 11   | DIM  | 1260+1604A>G (cryptic splice site) | Splicing | Ser421Leufs*4 | LOVD: NF1_000035 |
| 165 (1)   | 1     | 11   | SNV  | 1260+1G>A | Splicing | Ser421Ilefs*12 | LOVD: NF1_000036 |
| 289 (1)   | 1     | 12   | SNV  | 1261-2A>C (cryptic splice site) | Splicing | Ser421_Lys420del | LOVD: NF1_000045 |
| 82 (1)    | 1     | 12   | SNV  | 1318C>T  | Nonsense | Arg440* | LOVD: NF1_000052 |
| 153 (1)   | 4     | 12   | DEL  | 1329del | Frame-shift | Phe443Cysfs*30 | LOVD: NF1_001174 |
| 52 (1)    | 1     | 12   | INS  | 1378dup | Frame-shift | Ile460Asn*10 | New |
### Table A1. Cont.

| Family ID | Group | Exon | Type | Genomic | cDNA | Effect | Protein | ClinVar/HGMD/LOVD ID |
|-----------|-------|------|------|---------|------|--------|---------|----------------------|
| 21 (1)    | 1     | 12 (10a) | SNV  | 1381C>T | 1381C>T | Nonsense | Arg461* | LOVD: NF1_000056 |
| 57 (1)    | 3     | 12 (10a) | DIM  | 1393-1554C>GT (cryptic splice site) | 1392_1393insTGAGATTGTGTTACACACCATCCTACAAACATTAAACCCACGAGATGTGGCATATA | Intronic cryptic splice site | Ser465* | New |
| 144 (3)   | 4     | 166A>G (cryptic splice site) | 1466A>G (cryptic splice site) | 1466A>G (cryptic splice site) | 1466A>G (cryptic splice site) | Splicing | Ser465, Cys509del | LOVD: NF1_000063 |
| 160 (1)   | 1     | 12 (10a) | DIM  | 1393-1554C>GT (cryptic splice site) | 1392_1393insTGAGATTGTGTTACACACCATCCTACAAACATTAAACCCACGAGATGTGGCATATA | Intronic cryptic splice site | Ser465* | New |
| 17 (3)    | 1     | 13 (10b) | DEL  | 1393-2del | 1393_1527del | Splicing | Thr467Asnfs*3 | LOVD: NF1_002283 |
| 116 (1)   | 1     | 13 (10b) | INS  | 1399del | 1399del | Frame-shift | Lys476Asnfs*22 | New |
| 288 (1)   | 1     | 13 (10b) | DEL  | 1423del | 1423del | Splicing | Tyr489* | ClinVar: 354 |
| 13 (1)    | 4     | 13 (10b) | SNV  | 1466A>G (cryptic splice site) | 1466A>G (cryptic splice site) | Splicing | Tyr491Ilefs*9 | New |
| 222 (1)   | 1     | 13 (10b) | INS  | 1470_1471insATACG | 1470_1471insATACG | Splicing | Met496Arg | New |
| 132 (2)   | 1     | 13 (10b) | SNV  | 1487G>T | 1487G>T | Missense | Ile500_His501delinsLysAsn | New |
| 174 (1)   | 1     | 13 (10b) | INDEL | 1499_1501delinsAAA | 1499_1501delinsAAA | Splicing | Ile500_His501delinsLysAsn | New |
| 107 (1)   | 2     | 13 (10b) | DEL  | 1500del | 1500del | Frame-shift | His501Metfs*25 | New |
| 138 (1)   | 3     | 13 (10b) | DIM  | 1527+1165T>A (cryptic splice site) | 1527+1165T>A (cryptic splice site) | Splicing | Asn510Aspfs*7 | New |
| 76 (1)    | 1     | 14 (10c) | DEL  | 1541_1542del | 1541_1542del | Splicing | Gln514Argfs*43 | LOVD: NF1_000074 |
| 44 (3)    | 1     | 14 (10c) | SNV  | 1595T>G | 1595T>G | Frame-shift | Leu532Arg | LOVD: NF1_002496 |
| 5 (1)     | 4     | 15 (11) | SNV  | 1642-1G>A (splicing) | 1642-1G>A (splicing) | Splicing | Ala548Leufs*13 | LOVD: NF1_000084 |
| 147 (3)   | 5     | 15 (11) | SNV  | 1642G>T | 1642G>T | Splicing | Ala548Leufs*13 | New |
| 59 (1)    | 4     | 15 (11) | SNV  | 1658A>G | 1658A>G | Splicing | Ala548Leufs*13 | LOVD: NF1_000091 |
| 186 (1)   | 1     | 15 (11) | SNV  | 1721+3A>G (splicing) | 1721+3A>G (splicing) | Splicing | Ala548Leufs*13 | New |
| 85 (1)    | 1     | 15 (11) | SNV  | 1721G>C (splicing) | 1721G>C (splicing) | Splicing | Ala548Leufs*13 | New |
| 150 (1)   | 1     | 15-36 | DEL  | (infragenic deletion ex. 15-36) | 1624_4772+7del | Splicing | Ala548Valfs*9 | New |
| 15 (1)    | 1     | 16 (12a) | SNV  | 1748A>G (cryptic splice site) | 1748A>G (cryptic splice site) | Splicing | Ser574_Lys583delinsArg | LOVD: NF1_001100 |
| 102 (2)   | 1     | 17 (12b) | SNV  | 1846C>T | 1846C>T | Nonsense | Gln616* | LOVD: NF1_000125 |
| 133 (2)   | 1     | 17 (12b) | SNV  | 1942G>T | 1942G>T | Frame-shift | Ile679Glyfs*12 | LOVD: NF1_000148 |
| 168 (1)   | 2     | 17 (12b) | DEL  | 1863del | 1863del | Frame-shift | Cys622Valfs*9 | LOVD: NF1_001427 |
| 149 (1)   | 2     | 214 (1) | SNV  | 1942G>T | 1942G>T | Nonsense | Glu484* | LOVD: NF1_000248 |
| 75 (2)    | 1     | 17 (12b) | SNV  | 1995dup | 1995dup | Frame-shift | Ser666Leufs*4 | New |
| 217 (1)   | 1     | 18 (13) | SNV  | 2002-10T>A (cryptic splice site) | 2002-10T>A (cryptic splice site) | Splicing | Asp668Thrfs*23 | New |
| 8 (1)     | 1     | 18 (13) | SNV  | 2002-1G>A (cryptic splice site) | 2002-1G>A (cryptic splice site) | Splicing | Asp668Glnfs*17 | LOVD: NF1_000143 |
| 192 (1)   | 1     | 18 (13) | INDEL | 2027_2028delinsA | 2027_2028delinsA | Delsfs | Thr671Asnfs*12 | New |
| 39 (2)    | 1     | 18 (13) | INS  | 2033dup | 2033dup | Frame-shift | Ile679Aspfs*21 | LOVD: NF1_000148 |
| 63 (1)    | 1     | 18 (13) | SNV  | 2041C>T | 2041C>T | Nonsense | Arg681* | LOVD: NF1_000153 |
| 39 (2)    | 1     | 18 (13) | INS  | 2167dup | 2167dup | Frame-shift | Val723Glyfs*3 | New |
| 27 (2)    | 1     | 18 (13) | SNV  | 2251G>C (splicing) | 2251G>C (splicing) | Splicing | Asp668Glnfs*9 | ClinVar: 584927 |
| 209 (1)   | 1     | 19 (14) | SNV  | 2266C>T | 2266C>T | Splicing | Arg752Leufs*17 | LOVD: NF1_000174 |
| 75 (1)    | 1     | 19 (14) | SNV  | 2288T>C | 2288T>C | Missense | Leu763Pro | LOVD: NF1_000177 |
Table A1. Cont.

| Family ID | Group | Exon | Type | Genomic      | cDNA      | Effect       | Protein     | ClinVar/HGMD/LOVD ID |
|-----------|-------|------|------|--------------|-----------|--------------|-------------|----------------------|
| 7 (2)     | 1     | 19(14) | INS  | 2307dup      | 2307dup   | Frame-shift  | Thr770His*6   | New                  |
| 53 (1)    | 3     | 20(15) | SNV  | 2326G>A      | 2326_2409del | Splicing     | Trp777_Ala804del | New                  |
| 126 (1)   | 1     |       |      |              |           |              |             |                      |
| 184 (2)   | 1     | 20(15) | SNV  | 2339C>A      | 2339C>A   | Missense     | Thr780Lys    | LOVD: NF1_000190     |
| 234 (2)   | 1     | 20(15) | SNV  | 2339C>G      | 2339C>G   | Missense     | Thr780Arg    | LOVD: NF1_001397     |
| 6 (3)     | 1     | 20(15) | SNV  | 2351G>C      | 2351G>C   | Missense     | Trp784Ser    | LOVD: NF1_000196     |
| 33 (2)    | 1     | 20(15) | SNV  | 2352G>C      | 2352G>C   | Missense     | Trp784Cys    | LOVD: NF1_001853     |
| 106 (1)   | 4     | 21(16) | SNV  | 2540T>G      | 2540T>G   | Missense     | Leu847Arg    | LOVD: NF1_000229     |
| 188 (1)   | 1     |       |      |              |           |              |             |                      |
| 309 (1)   | 1     | 21(16) | SNV  | 2557C>T      | 2557C>T   | Nonsense     | Gln853*      | LOVD: NF1_001222     |
| 241 (1)   | 1     | 21(16) | SNV  | 2693T>C      | 2693T>C   | Missense     | Leu898Pro    | LOVD: NF1_000241     |
| 48 (1)    | 1     | 21(16) | SNV  | 2850+1G>A    | 2707_2850del | Splicing     | Cys904_Val951del | LOVD: NF1_000259     |
| 18 (1)    | 1     | 22(17) | SNV  | 2851G>T      | 2851_2990del | Splicing     | Leu952_Cysfs*22 | LOVD: NF1_001526     |
| 190 (2)   | 1     | 22(17) | DEL  | 2887C>T      | 2887C>T   | Nonsense     | Gln963*      | ClinVar: 233495      |
| 141 (1)   | 1     | 22(17) | DEL  | 2948del      | 2948del   | Frame-shift  | Leu983Glnfs*9 | New                  |
| 19 (2)    | 1     | 22(17) | DEL  | 2970_2972del | 2970_2972del | In-frame    | Met992del    | LOVD: NF1_000277     |
| 134 (1)   | 1     | 23(18) | SNV  | 3040A>T      | 3040A>T   | Non-sense    | Lys1014*     | ClinVar: 431616      |
| 140 (1)   | 4     | 23(18) | SNV  | 3104T>A      | 3104T>A   | Missense     | Met1035Lys   | New                  |
| 245 (1)   | 4     | 23(18) | SNV  | 3106A>G      | 3106A>G   | Missense     | Lys1036Glu   | New                  |
| 182 (1)   | 1     | 23(18) | SNV  | 3113+1G>A    | 2991_3113del | Splicing     | Tyr998_Arg1038del | LOVD: NF1_000306     |
| 230 (1)   | 3     | 25(19b) | SNV  | 3277G>A      | 3275_3314del | Splicing     | Gly1092Aspfs*7 | LOVD: NF1_000340     |
| 74 (1)    | 1     | 26(20) | SNV  | 3326T>G      | 3326T>G   | Non-sense    | Leu1109*     | New                  |
| 41 (1)    | 4     | 26(20) | DEL  | 3347_3350del | 3347_3350del | Frame-shift  | Asp1116Alafs*25 | LOVD: NF1_000351     |
| 255 (1)   | 1     |       |      |              |           |              |             |                      |
| 115 (1)   | 3     | 26(20) | SNV  | 3445A>G      | 3445A>G   | Missense     | Met1149Val   | LOVD: NF1_000356     |
| 20 (2)    | 1     | 26(20) | SNV  | 3496+1G>A    | 3315_3496del | Splicing     | Tyr1106Leufs*28 | HGMD: CS072245     |
| 40 (2)    | 5     | 27(21) | DEL  | 3502_3519del | 3502_3519del | In-frame    | Gly1168_Leu1173del | New                  |
| 284 (2)   | 6     | 27(21) | SNV  | 3592G>A      | 3592G>A   | Missense     | Glu1198Lys   | New                  |
| 215 (1)   | 1     | 27(21) | SNV  | 3610C>G      | 3610C>G   | Missense     | Arg1204Gly   | LOVD: NF1_000372     |
| 104 (1)   | 1     |       |      |              |           |              |             |                      |
| 166 (1)   | 1     |       |      |              |           |              |             |                      |
| 225 (1)   | 1     | 28-29 | DEL  | (3706+1_3709-1),(3972+1_3974-1)del | not determined | In-frame    | ?             | New                  |
| 275 (1)   | 3     | 29(23) | DEL  | 3899del      | 3899del   | Frame-shift  | Leu1301Profs*9 | New                  |
| 180 (1)   | 1     | 29(23) | SNV  | 3916C>T      | 3916C>T   | Non-sense    | Arg1306*     | LOVD: NF1_000416     |
| 58 (1)    | 1     | 29(23) | DEL  | 3972del      | 3972del   | Frame-shift  | Arg1325Glyfs*2 | New                  |
Table A1. Cont.

| Family ID 1 | Group | Exon | Type 2 | Genomic | cDNA 1 | Effect | Protein | ClinVar/HGMD/LOVD ID |
|-------------|-------|------|--------|---------|--------|--------|---------|----------------------|
| 247 (1)     | 1     | 29(23) | SNV    | 3974G>A | 3873_3976del | Splicing | Tyr1292Argfs*7 | LOVD: NF1_001992 |
| 129 (1)     | 1     | 30(23-1) | INS    | 4100_4103dup | 4100_4103dup | Frame-shift | Tyr1369Phefs*6 | New |
| 36 (1)      | 2     | 32(24) | DEL    | 4168del | 4168del | Frame-shift | Leu1390Serfs*17 | LOVD: NF1_000458 |
| 162 (1)     | 1     | 32(24) | SNV    | 4172G>C | 4172G>C | Missense | Arg1391Thr | LOVD: NF1_000461 |
| 285 (1)     | 1     | 32(24) | SNV    | 4219+2T>C | not determined | Splicing | ? | New |
| 261 (1)     | 5     | 33(25) | SNV    | 4276>G | 4276G>C | Missense | Gln1426Glu | LOVD: NF1_001275 |
| 154 (1)     | 1     | 33(25) | SNV    | 4279G>C | 4279G>C | Missense | Gln1426His | LOVD: NF1_000483 |
| 32 (4)      | 5     | 35(27a) | SNV    | 4515-21T>G(splicing) | 4514_4515insTTTGCTGTATCTAG | In-frame deletion | Tyr1512_Tyr1516del | LOVD: NF1_000586 |
| 16 (13)     | 1     | 33(25) | SNV    | 4515-2A | 4514_4515insTTTGCTGTATCTGG | In-frame deletion | Tyr1614_Tyr1618del | LOVD: NF1_001657 |
| 28 (1)      | 1     | 35(27a) | SNV    | 4537C>T | 4537C>T | Missense | Gln1426Asp | New |
| 9 (2)       | 1     | 35(27a) | SNV    | 4673C>A | 4673C>A | Missense | Arg1590Trp | HCMD: CM897051 |
| 181 (1)     | 1     | 35(27a) | DEL    | 4644del | 4644del | Frame-shift | Phe1548Leufs*5 | New |
| 66 (1)      | 1     | 36(27b) | DEL    | 4680_4683del | 4680_4683del | Frame-shift | Glu1561Asnfs*5 | New |
| 173 (1)     | 4     | 36(27b) | DEL    | 4691del | 4691del | Frame-shift | Lys1564Argfs*3 | New |
| 70 (1)      | 4     | 36(27b) | SNV    | 4768C>T | 4768C>T | Missense | Arg1590Trp | HCMD: CM897051 |
| 4 (1)       | 1     | 37(28) | SNV    | 4780del | 4780del | Frame-shift | Thr1594Leufs*9 | New |
| 119 (1)     | 2     | 37(28) | DEL    | 4840_4854del | 4840_4854del | In-frame deletion | Tyr1614_Tyr1618del | LOVD: NF1_001657 |
| 89 (1)      | 1     | 37(28) | DEL    | 4914_4917del | 4914_4917del | Frame-shift | Lys1640Gluysfs*36 | LOVD: NF1_000586 |
| 193 (1)     | 1     | 37(28) | SNV    | 4922G>A | 4922G>A | Non sense | Trp1641* | LOVD: NF1_001303 |
| 80 (2)      | 1     | 37(28) | DEL    | 4973_4978del | 4973_4978del | Non sense | Trp1641* | LOVD: NF1_000597 |
| 95 (2)      | 4     | 37-51 | DUP    | 5035-7,7426-7dup | not determined | Intra genetic duplication | ? | New |
| 10 (2)      | 1     | 38(29) | SNV    | 5264C>G | 5264C>G | Non sense | Ser1755* | HGMD: CM001260 |
| 1 (2)       | 4     | 38(29) | SNV    | 5401C>T | 5401C>T | Non sense | Gln1801* | LOVD: NF1_001390 |
| 101 (2)     | 5     | 38(29) | SNV    | 5425C>T | 5425C>T | Missense | Arg1809Cys | LOVD: NF1_000653 |
| 112 (5)     | 5     | 38(29) | SNV    | 5426G>C | 5426G>C | Missense | Arg1809Pro | ClinVar: 208835 |
| 178 (2)     | 5     | 38(29) | SNV    | 5426G>C | 5426G>C | Missense | Arg1809Leu | LOVD: NF1_000654 |
| 302 (1)     | 3     | 38(29) | SNV    | 5426G>C | 5426G>C | Missense | Arg1809Pro | ClinVar: 208835 |
| 155 (1)     | 4     | 38(29) | SNV    | 5426G>C | 5426G>C | Missense | Arg1809Leu | LOVD: NF1_000654 |
| 124 (3)     | 5     | 38(29) | SNV    | 5426G>C | 5426G>C | Missense | Arg1809Pro | ClinVar: 208835 |
| 156 (1)     | 4     | 38(29) | SNV    | 5426G>C | 5426G>C | Missense | Arg1809Leu | LOVD: NF1_000654 |
| 229 (1)     | 5     | 38(29) | SNV    | 5437T>C | 5437T>C | Missense | Ser1813Pro | New |
| 164 (2)     | 1     | 38(29) | SNV    | 5483A>T | 5483A>T | Missense | Asp1828Val | LOVD: NF1_000666 |
| 259 (1)     | 1     | 38(29) | SNV    | 5543T>A | 5543T>A | Missense | Lys1848* | LOVD: NF1_000670 |
| 163 (1)     | 1     | 39(30) | DEL    | 5592_5596del | 5592_5596del | Frame-shift | Asp1864lysfs*26 | New |
| 231 (1)     | 3     | 39(30) | SNV    | 5608C>T | 5608C>T | Non sense | Gln1870* | ClinVar: 237577 |
| 22 (1)      | 1     | 39(30) | SNV    | 5676G>T | 5676G>T | Missense | Lys1892Asn | New |
| 177 (3)     | 1     | 39(30) | SNV    | 5719G>T | 5719G>T | Non sense | Glu1967* | ClinVar: 187652 |
| 26 (2)      | 1     | 39(30) | DEL    | 5739del | 5739del | Frame-shift | Phe1913Leufs*8 | New |
| 24 (2)      | 1     | 40(31) | SNV    | 5839C>T | 5839C>T | Non sense | Arg1947* | LOVD: NF1_000711 |
| 171 (1)     | 1     | 40(31) | SNV    | 5842C>T | 5842C>T | Non sense | Gln1948* | LOVD: NF1_001913 |
Table A1. Cont.

| Family ID | Group | Exon | Type | Genomic | eDNA | Effect | Protein | ClinVar/HGMD/LOVD ID |
|-----------|-------|------|------|---------|------|--------|---------|---------------------|
| 151 (1)   | 1     | 40(31) | SNV | 5944-1G>C (cryptic splice site) | 5944-1G>C | Nonsense | Trp1976* | LOVD: NF1_002495 |
| 71 (1)    | 4     | 40(31) | SNV | 5944-5A>G (cryptic splice site) | 5944-5A>G | Nonsense | Gly1980Arg | ClinVar: 45773 |
| 282 (1)   | 3     | 41(32) | SNV | 6085-2A>T (splicing) | 6085-2A>T | Nonsense | Trp1976Cysfs*6 | ClinVar: 431977 |
| 34 (2)    | 1     | 42(33) | SNV | 6243C>A | 6243C>A | Nonsense | Y2081* | New |
| 83 (1)    | 1     | 42(33) | SNV | 6346+4A>G | 6346+4A>G | Nonsense | Val2091lysfs*7 | LOVD: NF1_001919 |
| 223 (1)   | 1     | 42(33) | SNV | 6352C>A | 6352C>A | Nonsense | Leu2112Pro | LOVD: NF1_000756 |
| 233 (1)   | 3     | 42(33) | SNV | 6580-2A>C | 6580-2A>C | Nonsense | Gly2125Glyfs*27 | HGMD: CS941517 |
| 97 (1)    | 3     | 43(34) | SNV | 6587+1G>T (splicing) | 6587+1G>T | Nonsense | Trp2204* | LOVD: NF1_000784 |
| 253 (1)   | 3     | 43(34) | SNV | 6606C>A | 6606C>A | Nonsense | Tyr2264* | LOVD: NF1_000815 |
| 42 (1)    | 3     | 44(35) | SNV | 6611G>A | 6611G>A | Nonsense | Cys2202* | LOVD: NF1_001338 |
| 137 (1)   | 1     | 44(35) | SNV | 6641+1G>C | 6641+1G>C | Nonsense | Ala2194Ilefs*6 | LOVD: NF1_000796 |
| 170 (1)   | 3     | 44(35) | SNV | 6686del | 6686del | Nonsense | Val2230Serfs*14 | LOVD: NF1_001670 |
| 139 (1)   | 1     | 45(36) | DEL | 6709C>T | 6709C>T | Nonsense | Arg2237* | LOVD: NF1_000802 |
| 50 (1)    | 1     | 45(36) | SNV | 6791_6792insAA | 6791_6792insAA | In-frame | Tyr2264* | LOVD: NF1_001349 |
| 73 (1)    | 1     | 46(37) | INS | 6791_6792insAA | 6791_6792insAA | Frame-shift | Tyr2264* | LOVD: NF1_000815 |
| 51 (2)    | 1     | 46(37) | INS | 6791dup | 6791dup | Frame-shift | Tyr2264* | LOVD: NF1_000815 |
| 118 (1)   | 3     | 46(37) | SNV | 6792C>A (STOP determining splicing) | 6792C>A | Splicing | Ala2253_Lys2286del | LOVD: NF1_000816 |
| 176 (1)   | 1     | 46(37) | SNV | 6792C>G (STOP determining splicing) | 6792C>G | Splicing | Ala2253_Lys2286del | LOVD: NF1_000817 |
| 299 (1)   | 3     | 46(37) | SNV | 6858+1G>T (splicing) | 6858+1G>T | Splicing | Ala2253_Lys2286del | LOVD: NF1_000824 |
| 30 (1)    | 1     | 46(37) | SNV | 6858+2T>C | 6858+2T>C | Splicing | Ala2253_Lys2286del | LOVD: NF1_000824 |
| 268 (1)   | 1     | 46(37) | SNV | 6881del | 6881del | Frame-shift | Leu2294Profs*4 | LOVD: NF1_001726 |
| 47 (1)    | 3     | 47(38) | DEL | 6898_6903del | 6898_6903del | In-frame | In-frame | New |
| 84 (1)    | 1     | 47(38) | DEL | 6955C>T | 6955C>T | Nonsense | Gln2195* | New |
| 88 (2)    | 1     | 47(38) | SNV | 6955C>T | 6955C>T | Nonsense | Gln2195* | New |
| 276 (1)   | 1     | 47(38) | SNV | 6974_6977del | 6974_6977del | Frame-shift | Asp2325Valfs*49 | LOVD: NF1_001352 |
| 94 (1)    | 1     | 48(39) | INS | 7089dup | 7089dup | Frame-shift | Asn2364* | LOVD: NF1_001359 |
| 183 (1)   | 1     | 48(39) | INS | 7125del | 7125del | Frame-shift | Tyr2277Thrfs*20 | LOVD: NF1_000849 |
| 43 (3)    | 1     | 48(39) | DEL | 7169_7170del | 7169_7170del | Frame-shift | Arg2390Lysfs*10 | New |
| 158 (3)   | 1     | 48(39) | DEL | 7184T>C | 7184T>C | Frame-shift | Leu2395Pro | LOVD: NF1_000857 |
| 169 (1)   | 1     | 49(40) | DEL | 7232dup | 7232dup | Frame-shift | Asn2411Lysfs*16 | New |
| 79 (2)    | 1     | 49(40) | SNV | 7232dup | 7232dup | Frame-shift | Asn2411Lysfs*16 | New |
| 227 (1)   | 1     | 49(40) | INS | 7259C>A | 7259C>A | Nonsense | Ala2420Asp | LOVD: NF1_000867 |
| 185 (1)   | 1     | 50(41) | SNV | 7285C>T | 7285C>T | Nonsense | Arg2449* | LOVD: NF1_000871 |
| 3 (1)     | 1     | 50(41) | SNV | 7518del | 7518del | Frame-shift | Gly2518_Met2558del | ClinVar: 237598 |
| 87 (1)    | 1     | 51(42) | DEL | 7518del | 7518del | Frame-shift | Gly2518_Met2558del | ClinVar: 237598 |
| 37 (1)    | 3     | 51(42) | DEL | 7533_7567del | 7533_7567del | In-frame | In-frame | New |
| 130 (1)   | 4     | 52(43) | SNV | not determined | not determined | Frame-shift | Ile2563Phefs*40 | LOVD: NF1_002529 |
| 12 (2)    | 1     | 53(44) | DEL | 7686del | 7686del | Frame-shift | Ser2626Profs*33 | New |
| 25 (2)    | 1     | 54(45) | INS | 7874_7875dup | 7874_7875dup | Frame-shift | Ser2626Profs*33 | New |
**Table A1. Cont.**

| Family ID  | Group | Exon | Type  | Genomic     | cDNA     | Effect     | Protein                  | ClinVar/HGMD/LOVD ID |
|------------|-------|------|-------|-------------|----------|------------|--------------------------|----------------------|
| 103 (1)    | 1     | 56(47)| SNV   | 8051-1G>C   | 8051_8097del | Splicing   | Ser2684Thrfs*9           | New                  |
| 260 (1)    | 4     | 57(48)| INS   | 8207_8231dup| 8207_8231dup | Frame-shift | Leu2745Serfs*14         | New                  |
| 61 (1)     | 2     |      |       |             |          |            |                          |                      |
| 64 (1)     | 2     |      |       |             |          |            |                          |                      |
| 69 (1)     | 2     |      |       |             |          |            |                          |                      |
| 77 (1)     | 2     |      |       |             |          |            |                          |                      |
| 78 (1)     | 1     | all  | DEL   | -718-?_8375+?del | Microdeletion 17q11.2 | Microdeletion 17q11.2 | ? | LOVD: NF1_000001 |
| 79 (1)     | 2     |      |       |             |          |            |                          |                      |
| 101 (1)    | 2     | 1    |       |             |          |            |                          |                      |
| 127 (1)    | 2     |      |       |             |          |            |                          |                      |
| 136 (1)    | 1     |      |       |             |          |            |                          |                      |
| 171 (1)    | 1     |      |       |             |          |            |                          |                      |
| 277 (1)    | 1     |      |       |             |          |            |                          |                      |

1 Number of family members presenting the variant is reported in parentheses. 2 Type of variant: SNV = Single-nucleotide variant, DEL = Deletion, DUP = Duplication, INS = Insertion, INDEL = Insertion-deletion, DIM = Deep intronic mutation. 3 ID of annotated variants in ClinVar (www.ncbi.nlm.nih.gov/clinvar), Human Genome Variation Database (HGMD; www.hgmd.cf.ac.uk), and Leiden Open Variation Database (LOVD; databases.lovd.nl/shared/genes/NF1).
### Table A2. Unique variants identified in *SPRED1* (RefSeq NM_152594.2).

| Family ID | Group | Exon | Type | Genomic | cDNA | Effect | Protein | ClinVar/HGMD/LOVD ID |
|-----------|-------|------|------|---------|------|--------|---------|----------------------|
| 11(10)    | 5     | 2    | DEL  | 49,53del| 49,53del| Frame-shift Val17Serfs*8 | New |
| 224(1)    | 3     | 2    | SNV  | 52C>T   | 52C>T   | Nonsense Arg18* | LOVD: SPRED1_000121 |
| 92(3)     | 5     | 2    | SNV  | 70C>T   | 70C>T   | Nonsense Arg24* | LOVD: SPRED1_000014 |
| 179(3)    | 5     | 2    | SNV  | 74A>G   | 74A>G   | Missense Asp25Gly | ClinVar: 391600 |
| 161(2)    | 5     | 3    | SNV  | 229A>T  | 229A>T  | Nonsense Lys77* | LOVD: SPRED1_000112 |
| 157(1)    | 4     | 6–7  | DEL  | *618-*691+del | *618-*691+del | Nonsense Arg325* | New |
| 167(4)    | 5     | 7    | SNV  | 973C>T  | 973C>T  | Nonsense Arg325* | LOVD: SPRED1_000077 |
| 286(2)    | 5     | 7    | DUP  | 993dup  | 993dup  | Frame-shift Arg332Thrfs*12 | New |

1. Number of family members presenting the variant is reported in parentheses.
2. Type of variant: SNV = Single-nucleotide variant, DEL = Deletion, DUP = Duplication.
3. ID of annotated variants in ClinVar (www.ncbi.nlm.nih.gov/clinvar), Human Genome Variation Database (HGMD; www.hgmd.cf.ac.uk), and Leiden Open Variation Database (LOVD; databases.lovd.nl/shared/genes/SPRED1).
Table A3. Clinical features of 245 probands with suspicion of NF1 or an NF1-like condition (the most serious clinical features that could reduce patients' life expectancy are highlighted in bold).

| Group | Family ID | Patient ID | Sex | Molecularily Characterized Affected Relatives | Sporadic Y/N | Paternal/Maternal Inheritance | Age (yy:mm) | CALMs (≥6) | Freckling | Lisch Nodules | OPG | Bone Dysplasia | Neurofibromas (Cutaneous or Plexiform) | Other Clinical Features |
|-------|-----------|------------|-----|-----------------------------------------------|--------------|-----------------------------|-------------|-----------|-----------|---------------|-----|----------------|---------------------------------------|------------------------|
| 1     | 2         | 0 F        | N   | N Maternal                                     |              | 2.00                        | 12:00       | +         | +         | +             | +   | -             | +                                     | LGG, DLL               |
| 1     | 3         | 5 M        | N   | N Maternal                                     | 6.08         | 14:00                       | 18:05       | +         | +         | +             | +   | -             | +                                     | Thor                   |
| 1     | 4         | 6 M        | N   | N Paternal                                     | 14.02        | 18:05                       | -           | +         | +         | -             | -   | -             | +                                     | DLL, DS, ID             |
| 1     | 5         | 7 M        | N   | N Paternal                                     | 6.13         | 22:00                       | -           | +         | +         | +             | +   | -             | +                                     | NBOs, Epy               |
| 1     | 6         | 7 M        | N   | N Paternal                                     | 10.19:00     | 19:00                       | +           | +         | +         | +             | -   | -             | +                                     | DLL                    |
| 1     | 7         | 8 M        | N   | N Paternal                                     | 14.09        | 17:00                       | -           | +         | +         | +             | +   | -             | +                                     | UH                     |
| 1     | 8         | 9 M        | N   | N Paternal                                     | 10.15:04     | 15:04                       | +           | +         | +         | +             | +   | -             | +                                     | NBOs                   |
| 1     | 9         | 10 M       | N   | N Paternal                                     | 14.08        | 14:08                       | +           | +         | +         | +             | +   | -             | +                                     | LD, SP                  |
| 1     | 10        | 12 M       | N   | N Paternal                                     | 10.01        | 12:04                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 11        | 14 M       | Y   | N Paternal                                     | 9.09         | 11:08                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 12        | 15 M       | N   | N Maternal                                     | 10.11        | 11:01                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 13        | 16 M       | N   | N Maternal                                     | 10.01        | 12:04                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 14        | 17 M       | N   | N Paternal                                     | 3.01         | 9:01                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 15        | 18 M       | N   | N Paternal                                     | 5.09         | 8:11                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 16        | 19 M       | Y   | N Paternal                                     | 12.01        | 15:08                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 17        | 20 M       | N   | N Paternal                                     | 11.19        | 9:01                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 18        | 21 M       | N   | N Paternal                                     | 21.11        | 22:08                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 19        | 22 M       | N   | N Paternal                                     | 14.08        | 15:06                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 20        | 23 M       | Y   | N Paternal                                     | 15.06        | 17:06                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 21        | 24 M       | Y   | N Paternal                                     | 13.09        | 17:06                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 22        | 25 M       | N   | N Maternal                                     | 11.02        | 11:07                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 23        | 26 M       | Y   | N Maternal                                     | 6.01         | 10:00                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 24        | 27 M       | N   | N Paternal                                     | 15.03        | 18:11                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 25        | 28 M       | N   | N Maternal                                     | 7.00         | 8:11                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 26        | 29 M       | N   | N Paternal                                     | 19.04        | 22:11                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 27        | 30 M       | N   | N Paternal                                     | 11.09        | 13:01                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 28        | 31 M       | N   | N Paternal                                     | 9.07         | 10:03                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 29        | 32 M       | N   | N Paternal                                     | 14.02        | 14:08                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 30        | 33 M       | N   | N Paternal                                     | 14.05        | 16:00                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 31        | 34 M       | N   | N Paternal                                     | 22.27        | 27:00                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 32        | 35 M       | N   | N Paternal                                     | 6.04         | 7:05                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 33        | 36 M       | N   | N Paternal                                     | 8.06         | 9:05                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 34        | 37 M       | N   | N Paternal                                     | 7.08         | 8:01                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 35        | 38 M       | Y   | N Paternal                                     | 35.36        | 36:06                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 36        | 39 M       | Y   | N Paternal                                     | 14.18        | 18:00                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 37        | 40 M       | Y   | N Paternal                                     | 20.01        | 21:09                       | +           | +         | +         | +             | +   | -             | +                                     |                |
| 1     | 38        | 41 M       | Y   | N Paternal                                     | 4.06         | 6:06                        | +           | +         | +         | +             | +   | -             | +                                     |                |
| Group | Family Patient ID | Sex | Molecularly Characterized Affected Relatives | Sporadic (Y/N) | Paternal/Maternal Inheritance | Age (yy:mm) | CALMs (≥6) | Freckling | Lisch Nodules | OPG | Bone Dysplasia | Neurofibromas (Cutaneous or Plexiform) | Other Clinical Features |
|-------|------------------|-----|---------------------------------------------|---------------|-------------------------------|-------------|-----------|----------|--------------|-----|--------------|--------------------------------------|-----------------------|
| 1     | 51 102 F         | 1   | N Maternal                                   | 5             | 7:08                          | +           | +         | +        | +            | +   | -            | +                                    | -                     |
| 1     | 52 104 F         | 0   | Y                                            | 13            | 14:02                         | +           | +         | +        | +            | +   | -            | +                                    | BvP, NA, LD, SP, CD   |
| 1     | 55 107 M         | (twin) | Y                                            | 9:02          | 14:04                         | +           | +         | +        | +            | +   | -            | +                                    | DLL, NBos              |
| 1     | 58 112 F         | 0   | N Maternal                                   | 7             | 10                            | +           | +         | +        | +            | +   | n.a.         | +                                    | mild ID               |
| 1     | 63 125 F         | 0   | Y                                            | 16:04         | 16:07                         | +           | +         | +        | +            | +   | -            | +                                    | DLL, NBos, Thor, MacroC|
| 1     | 65 128 F         | 0   | Y                                            | 12            | 19:02                         | +           | +         | +        | +            | +   | -            | +                                    | LD, DS, NBos, Thor     |
| 1     | 66 129 M         | 0   | Y                                            | 7             | 13:03                         | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 67 130 M         | 0   | Y                                            | 12            | 13:03                         | +           | +         | +        | n.a.         | n.a.| -            | +                                    | DLL, NBos              |
| 1     | 68 132 M         | 0   | Y                                            | 7             | 13:06                         | +           | -         | +        | -            | +   | -            | +                                    |                      |
| 1     | 72 137 M         | 0   | Y                                            | 21            | 22:02                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 73 138 F         | 0   | Y                                            | 17:03         | 19:05                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 74 139 F         | 0   | Y                                            | 12:04         | 13:04                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 75 140 M         | 0   | N Maternal                                   | 6:05          | 8:02                          | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 76 141 F         | 0   | Y                                            | 9:09          | 12:03                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 78 149 F         | 0   | Y                                            | 16            | 18                            | +           | n.a.      | +        | -            | +   | -            | +                                    |                      |
| 1     | 79 151 M         | 1   | N Maternal                                   | 5:06          | 8:05                          | +           | +         | +        | -            | +   | -            | +                                    | -                     |
| 1     | 80 155 M         | 1   | N Paternal                                   | 4:03          | 5:05                          | +           | +         | -        | -            | +   | -            | +                                    | NBos, ID               |
| 1     | 82 158 M         | 0   | N Maternal                                   | 4:11          | 5:11                          | +           | +         | -        | -            | +   | -            | +                                    | DLL                   |
| 1     | 83 160 F         | 0   | Y                                            | 8             | 9:07                          | +           | +         | +        | +            | +   | -            | +                                    | NBos, BvP              |
| 1     | 84 161 F         | 0   | Y                                            | 23:01         | 25:11                         | +           | +         | +        | +            | +   | -            | +                                    | NBos                   |
| 1     | 85 162 F         | 0   | N Paternal                                   | 1:11          | 3:01                          | +           | +         | +        | -            | +   | -            | +                                    | MacroC, IU             |
| 1     | 87 171 F         | 0   | N Paternal                                   | 7             | 8:01                          | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 88 253 F         | 1   | N Maternal                                   | 7:06          | 8:06                          | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 89 175 F         | 0   | Y                                            | 20            | 21                            | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 93 185 M         | 2   | N Maternal                                   | 7:06          | 11                            | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 94 187 M         | 0   | N Maternal                                   | 15            | 18:04                         | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 98 204 F         | 0   | N Paternal                                   | 13:06         | 16:06                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 102 218 F        | 1   | N Maternal                                   | 25            | 27                            | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 103 220 F        | 0   | Y                                            | 21            | 23                            | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 104 223 F        | 0   | Y                                            | 15            | 16:05                         | +           | +         | n.a.     | -            | +   | -            | +                                    | -                     |
| 1     | 108 228 M        | 0   | Y                                            | 0:05          | 3:05                          | +           | +         | +        | -            | +   | -            | +                                    | -                     |
| 1     | 109 236 M        | 1   | N Maternal                                   | 12:07         | 13:01                         | +           | +         | +        | +            | +   | -            | +                                    | NA, MacroC, Thor, PmD  |
| 1     | 114 247 M        | 0   | Y                                            | 18            | 21:08                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 116 250 M        | 0   | Y                                            | 12:03         | 13:03                         | +           | +         | +        | -            | +   | -            | +                                    |                      |
| 1     | 126 271 M        | 0   | N Maternal                                   | 4:05          | 5:03                          | +           | +         | -        | +            | +   | -            | +                                    | UH, NBos, BvP, LD      |
| 1     | 129 276 M        | 0   | Y                                            | 6:04          | 7                             | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 132 286 M        | 1   | N Paternal                                   | 11:09         | 12:01                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 133 287 F        | 1   | N n.a.                                       | 1             | 1:06                          | +           | +         | +        | -            | +   | -            | +                                    | Noon                  |
| 1     | 134 289 M        | 0   | Y                                            | 10            | 10:08                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 136 291 F        | 0   | Y                                            | 15:05         | 15:09                         | +           | +         | +        | +            | +   | -            | +                                    | LD, PmD, SD, NBos, BvP, SF |
| 1     | 137 309 F        | 0   | Y                                            | 0:08          | 1:01                          | +           | +         | -        | -            | +   | -            | +                                    |                      |
| 1     | 139 301 M        | 1   | N n.a.                                       | 30:06         | 31:02                         | +           | +         | +        | +            | +   | -            | +                                    |                      |
| 1     | 144 317 F        | 2   | N Maternal                                   | 30            | 30:03                         | +           | +         | +        | +            | +   | -            | +                                    | MacroC, Noon           |
| Group | Family ID | Patient ID | Sex | Molecularly Characterized Affected Relatives | Sporadic (Y/N) | Paternal/Maternal Inheritance | Age (mm) | CALMs (>6) Freckling | Lisch Nodules | OPG Bone Dysplasia (Cutaneous or Plexiform) | Neurofibromas (Cutaneous or Plexiform) | Other Clinical Features |
|-------|-----------|------------|-----|---------------------------------------------|---------------|--------------------------------|----------|----------------------|-------------|------------------------|----------------------------|------------------------|
| 1     | 150       | 325        | M   | 0                                           | Y             |                                 | 11:11    | +                    | +           | +                      | +                         | +                      | BSL, LD                 |
| 1     | 151       | 326        | F   | 0                                           | Y             |                                 | 14:01    | +                    | +           | +                      | +                         | +                      | MMS                    |
| 1     | 152       | 328        | M   | 0                                           | Y             |                                 | 13:06    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 153       | 329        | M   | 0                                           | Y             |                                 | 19:19    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 154       | 331        | M   | 0                                           | Y             |                                 | 20:06    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 158       | 341        | F   | 2                                           | N             | Maternal                       | 15:03    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 159       | 343        | M   | 0                                           | Y             |                                 | 14:11    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 160       | 385        | M   | 0                                           | Y             |                                 | 29:01    | +                    | -           | +                      | -                         | -                      |                       |
| 1     | 162       | 354        | F   | 0                                           | Y             |                                 | 42:06    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 163       | 350        | F   | 0                                           | Y             |                                 | 49:01    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 164       | 384        | F   | 1                                           | N             | Paternal                       | 12:06    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 165       | 426        | F   | 0                                           | N             | Paternal                       | 20:26    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 166       | 340        | M   | 0                                           | Y             |                                 | 17:01    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 167       | 334        | M   | 0                                           | Y             |                                 | 18:19    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 171       | 405        | M   | 0                                           | Y             |                                 | 5:05:01  | +                    | -           | +                      | +                         | +                      |                       |
| 1     | 172       | 395        | M   | 0                                           | Y             |                                 | 37:02    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 174       | 435        | F   | 0                                           | Y             |                                 | 60:02    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 176       | 463        | F   | 0                                           | Y             |                                 | 37:03    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 177       | 199        | M   | 2                                           | N             | Paternal                       | 10:03    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 180       | 340        | M   | 0                                           | Y             |                                 | 13:15    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 181       | 450        | F   | 0                                           | Y             |                                 | 15:01    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 182       | 95         | M   | 0                                           | Y             |                                 | 37:21    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 183       | 146        | M   | 0                                           | Y             | Paternal                       | 15:17    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 184       | 166        | M   | 1                                           | N             | Maternal                       | 24:31    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 185       | 146        | M   | 0                                           | Y             |                                 | 69:76    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 186       | 189        | F   | 0                                           | Y             |                                 | 28:34    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 187       | 255        | M   | 0                                           | Y             |                                 | 41:42    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 188       | 367        | F   | 1                                           | N             | Paternal                       | 68:70    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 189       | 425        | M   | 0                                           | Y             |                                 | 20:22    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 190       | 431        | M   | 0                                           | Y             |                                 | 31:36    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 191       | 86         | F   | 0                                           | Y             |                                 | 20:20    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 192       | 345        | M   | 0                                           | Y             |                                 | 4:6      | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 193       | 432        | M   | 0                                           | Y             |                                 | 33:33    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 194       | 442        | M   | 0                                           | Y             |                                 | 34:36:8  | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 195       | 453        | F   | 0                                           | Y             |                                 | 13:14:5  | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 196       | 470        | F   | 0                                           | Y             |                                 | 20:21    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 197       | 710        | F   | 0                                           | Y             |                                 | 7:09     | +                    | -           | -                      | -                         | +                      |                       |
| 1     | 198       | 471        | M   | 0                                           | Y             |                                 | 19:19    | +                    | +           | +                      | -                         | -                      |                       |
| 1     | 199       | 481        | M   | 0                                           | Y             |                                 | 10:02    | +                    | -           | -                      | -                         | +                      |                       |
| 1     | 201       | 483        | M   | 0                                           | N             |                                 | 0:1:6    | +                    | -           | -                      | -                         | -                      |                       |
| 1     | 202       | 491        | M   | 0                                           | Y             |                                 | 37:40    | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 203       | 495        | F   | 0                                           | Y             |                                 | 8:10:01  | +                    | +           | +                      | +                         | +                      |                       |
| 1     | 204       | 500        | M   | 0                                           | Y             |                                 | 13:13    | +                    | +           | +                      | +                         | +                      |                       |
Table A3. Cont.

| Group | Family ID | Patient ID | Sex | Molecularly Characterized Affected Relatives | Sporadic (Y/N) | Paternal/Maternal Inheritance | Age (yy:mm) | CALMs (≥6) | Freckling | Lisch Nodules | OPG | Bone Dysplasia | Neurofibromas (Cutaneous or Plexiform) | Other Clinical Features |
|-------|-----------|------------|-----|---------------------------------------------|---------------|-----------------------------|------------|-----------|-----------|--------------|-----|----------------|--------------------------------------|------------------------|
| 1     | 233       | 523        | F   | 0                                           |               |                             | 21 21:05   | +         | -         | -            | -   | -              | +                                    | Hyd, NA, Noon, Chem, SS |
| 1     | 234       | 524        | M   | 1                                           | N             | Paternal                    | 17 18:2    | +         | +         | +            | -   | -              | +                                    | NBos, LD, DS, BP |
| 1     | 241       | 548        | M   | 0                                           | N             | Maternal                    | 30 33      | +         | +         | +            | -   | -              | +                                    | NBos, LD |
| 1     | 247       | 567        | M   | 0                                           | N             | Maternal                    | 5 7        | +         | +         | +            | -   | -              | +                                    | NBos |
| 1     | 251       | 575        | M   | 0                                           | Y             |                             | 10 11      | +         | +         | +            | +   | +              | -                                    | ID, NBos |
| 1     | 255       | 585        | M   | 0                                           | Y             |                             | 12 13.03   | +         | -         | -            | -   | -              | -                                    | ID |
| 1     | 256       | 592        | M   | 0                                           | Y             |                             | 19 20      | +         | +         | -            | +   | -              | +                                    | ID |
| 1     | 259       | 602        | M   | 0                                           | Y             |                             | 13 14:25   | +         | +         | +            | +   | +              | +                                    | AD |
| 1     | 268       | 632        | M   | 0                                           | Y             |                             | 15 17      | +         | -         | -            | -   | -              | +                                    | ID |
| 1     | 276       | 646        | M   | 0                                           | Y             |                             | 1 30:05    | +         | +         | +            | +   | +              | -                                    | ID |
| 1     | 277       | 647        | M   | 0                                           | Y             |                             | 14 18:2    | +         | +         | +            | +   | +              | -                                    | ID |
| 1     | 285       | 681        | M   | 0                                           | Y             |                             | 51 54      | +         | +         | +            | +   | +              | +                                    | ID |
| 1     | 288       | 70         | M   | 0                                           | N             | Maternal                    | 10 11:11   | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 36        | 70         | M   | 0                                           | N             | Maternal                    | 10 12:11   | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 61        | 118        | M   | 0                                           | Y             |                             | 2:01 3:11  | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 64        | 127        | M   | 0                                           | Y             |                             | 14:06 16:02| +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 69        | 134        | M   | 0                                           | Y             |                             | 16 17:02   | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 77        | 147        | M   | 0                                           | Y             |                             | 4:09 6:06  | +         | +         | -            | -   | -              | +                                    | ID |
| 2     | 99        | 205        | M   | 0                                           | Y             |                             | 29 29:08   | +         | +         | -            | -   | -              | +                                    | ID |
| 2     | 107       | 227        | M   | 0                                           | Y             |                             | 5 7        | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 119       | 229        | M   | 0                                           | Y             |                             | 11:02 13   | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 127       | 272        | M   | 0                                           | Y             |                             | 15 15:09   | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 149       | 324        | M   | 0                                           | Y             |                             | 14 16      | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 166       | 97         | M   | 0                                           | N             | Paternal                    | 20 23      | +         | +         | +            | +   | +              | +                                    | ID |
| 2     | 3    | 71         | M   | 0                                           | Y             |                             | 9 12:11    | +         | +         | +            | +   | +              | +                                    | Leuk |
| 2     | 42        | 83         | M   | 0                                           | Y             |                             | 6 8:09     | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 45        | 92         | F   | 0                                           | Y             |                             | 9:02 14:08 | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 47        | 96         | F   | 0                                           | Y             |                             | 4:11 5:11  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 49        | 100        | M   | 0                                           | Y             |                             | 1:04 3:01  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 53        | 105        | M   | 0                                           | Y             |                             | 8 10       | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 57        | 110        | F   | 0                                           | Y             |                             | 3:09 4:03  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 81        | 157        | M   | 0                                           | Y             |                             | 2:01 4:11  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 91        | 167        | M   | 0                                           | Y             |                             | 3:05 4:07  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 92        | 203        | F   | 0                                           | Y             |                             | 3 7:2      | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 110       | 238        | F   | 0                                           | Y             |                             | 1:01 4:02  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 113       | 245        | F   | 0                                           | Y             |                             | 9:01 12:02 | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 115       | 249        | F   | 0                                           | Y             |                             | 9 9:02     | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 118       | 257        | M   | 0                                           | Y             |                             | 5:08 8     | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 120       | 260        | F   | 0                                           | Y             |                             | 8:08 12    | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 121       | 261        | F   | 0                                           | Y             |                             | 1:09 3:05  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 125       | 268        | F   | 0                                           | Y             |                             | 3:05 3:06  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 128       | 273        | M   | 0                                           | Y             |                             | 8:08 10:04 | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 131       | 282        | F   | 0                                           | Y             |                             | 4 9:01     | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 135       | 290        | F   | 0                                           | Y             |                             | 9:03 10:01 | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| 2     | 138       | 293        | M   | 0                                           | Y             |                             | 5:08 5:01  | +         | +         | +            | +   | +              | +                                    | Thor, DS, DLL |
| Group | Family Patient ID | Sex | Molecularly Characterized Affected Relatives | Sporadic (Y/N) | Paternal/Maternal Inheritance | Age (yr:mm) | CALMs (≥6) | Freckling | Lisch Nodules | OPG | Bone Dysplasia | Neurofibromas (Cutaneous or Plexiform) | Other Clinical Features |
|-------|------------------|-----|---------------------------------------------|---------------|-------------------------------|-------------|------------|----------|-------------|-----|--------------|------------------------------------|------------------------|
| 3     | 142 314 M        | Y   | 0                                           | Y             | 8.06 10                       | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 145 321 F        | Y   | 0                                           | Y             | 9.01 10.01                    | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 146 322 F        | Y   | 0                                           | Y             | 6.07 7                        | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 170 145 M        | Y   | 0                                           | Y             | 3.05 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 175 358 F        | Y   | 0                                           | Y             | 2.08 4.04                     | + +         | -          | +        | -           | -   | -            | -                                  |                        |
| 3     | 202 374 M        | Y   | 0                                           | Y             | 8.09 6.07                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 215 464 F        | Y   | 0                                           | Y             | 2.30 3.03                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 224 494 M        | Y   | 0                                           | Y             | 9.12 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 228 503 M        | Y   | 0                                           | Y             | 2.30 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 230 507 M        | Y   | 0                                           | Y             | 7.83 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 231 508 F        | Y   | 0                                           | Y             | 1.03 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 239 536 M        | Y   | 0                                           | Y             | 5.03 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 244 555 M        | Y   | 0                                           | Y             | 7.01 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 252 576 M        | Y   | 0                                           | Y             | 3.5 6.02                      | + +         | +          | -        | -           | -   | -            | -                                  |                        |
| 3     | 253 579 M        | Y   | 0                                           | Y             | 7.09 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 275 644 M        | Y   | 0                                           | Y             | 6.07 6.02                     | + +         | +          | -        | -           | -   | -            | -                                  |                        |
| 3     | 278 649 M        | Y   | 0                                           | Y             | 3.08 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 279 650 M        | Y   | 0                                           | Y             | 2.30 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 282 653 M        | Y   | 0                                           | Y             | 8.06 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 294 672 F        | Y   | 0                                           | Y             | 2.10 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 297 677 F        | Y   | 0                                           | Y             | 7.08 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 299 680 M        | Y   | 0                                           | Y             | 6.08 6.02                     | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 3     | 302 683 M        | Y   | 0                                           | Y             | 1.2 6.02                      | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 1 3 M            | Y   | 1 (twin)                                    | Y             | 12.03 17                      | + +         | +          | +        | +           | n.a.| n.a.        | -                                  | LD                    |
| 4     | 5 7 M            | Y   | 0                                           | Y             | 10.07 12.08                   | + +         | -          | -        | -           | +   | -            | -                                  |                        |
| 4     | 13 20 M          | Y   | 0                                           | Y             | 16.18 16.02                   | + +         | +          | +        | -           | -   | -            | -                                  |                        |
| 4     | 41 82 F          | Y   | 0                                           | Y             | 12.03 13.01                   | + +         | +          | +        | -           | -   | -            | -                                  |                        |
| 4     | 54 106 M         | Y   | 0                                           | Y             | 15.04 16.02                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 56 109 M         | Y   | 0                                           | Y             | 11.03 13.05                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 59 113 F         | Y   | 0                                           | Y             | 17.06 18                      | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 70 135 M         | Y   | 0                                           | Y             | 15.07 17.05                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 117 251 M        | Y   | 0                                           | Y             | 13.04 14.04                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 123 264 M        | Y   | 0                                           | Y             | 11.14 14.07                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 130 278 M        | Y   | 0                                           | Y             | 15.07 18.01                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 140 306 F        | Y   | 0                                           | Y             | 14.01 14.08                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 143 315 F        | Y   | 0                                           | Y             | 14.09 12.04                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 155 339 F        | Y   | 0                                           | Y             | 11.09 12.04                   | + +         | -          | -        | -           | n.a.| n.a.        | -                                  |                        |
| 4     | 156 195 F        | Y   | 0                                           | Y             | 11.09 12.04                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
| 4     | 157 196 F        | Y   | 0                                           | Y             | 11.09 12.04                   | + +         | -          | -        | -           | -   | -            | -                                  |                        |
Table A3. Cont.

| Group |
|-------|
| 4     |
| 5     |
| 6     |
| 7     |
| 8     |

| Family ID  | Patient ID  | Sex | Molecularly Characterized Affected Relatives | Sporadic (Y/N) | Age (yy:mm) | CALMs (≥6) | Freckling | Lisch Nodules | OPG | Bone Dysplasia | Neurofibromas (Cutaneous or Plexiform) | Other Clinical Features |
|-----------|-------------|-----|-----------------------------------------------|----------------|-------------|------------|-----------|---------------|-----|---------------|--------------------------------------|------------------------|
| 173       | 404         | F   | 0                                             | Y              | 15.01 15.07 | +          | + +       | -             | -   | -             | -                                    | Ss, MacroC              |
| 196       | 87          | F   | 0                                             | Y              | 14 11      | + +        | -         | -             | -   | -             | -                                    | OD                     |
| 201       | 265         | M   | 0                                             | Y              | 13 14      | + +        | -         | -             | -   | -             | -                                    | NA                     |
| 227       | 532         | M   | 0                                             | Y              | 13 14      | + +        | -         | -             | -   | -             | -                                    | NA                     |
| 245       | 557         | F   | 0                                             | Y              | 10 11.05   | + +        | -         | -             | -   | -             | -                                    | NA                     |
| 260       | 608         | F   | 0                                             | Y              | 34 36      | + +        | +         | -             | -   | -             | -                                    | NA                     |
| 269       | 635         | M   | 0                                             | Y              | 14 15      | + +        | -         | -             | -   | -             | -                                    | NA                     |
| 280       | 651         | F   | 0                                             | Y              | 14 15      | + +        | -         | -             | -   | -             | -                                    | NA                     |
| 281       | 652         | M   | 0                                             | Y              | 10 12      | + +        | -         | -             | -   | -             | -                                    | NA                     |
| 296       | 675         | F   | 0                                             | Y              | 29 29      | + +        | -         | -             | -   | -             | -                                    | NA                     |

1 The most serious clinical features that could reduce patients’ life expectancy are highlighted in bold. Abbreviations: AD = Attention deficit; ArC = Arachnoid cyst; AIC = Atlantal CALMs; AtIN = Atypical iris nodules; Bover = Bone overgrowth; BP = Bilateral ptosis; BvP = Brain stem lesions; CD = Other cardiac defects (DIA, HCM, CoA); Chem = Cherry hemangioma; Clm = Chiari I malformation; Cry = Cryptorchidism; DE = Dural ectasia; Diffuse lipomas; DLL = Legs of different length (>1 cm); DS = Dystrophic scoliosis; Epv = Epilepsy (idiopathic); HY = Hypertension; Id = Intellectual disability (moderate or severe); IH = Inguinal herniation; IPP = Idiopathic precocious puberty; LD = Learning disabilities (calculation, reading, memory); Leuk = Leukemia; LGG = Low-grade glioma (other than OPG); MacroC = Macrocephaly (>95th PCTL or +3.33 plus 2 SD of difference with height); MBG = Multifocal brain gliomas; MMS = Moyamoya syndrome; MPNST = Malignant peripheral nerve sheath tumor; Myo = Myopia; NA = Nevus anemicus; NBOs = Neurofibromatosis bright objects; OD = Oligodontia; OPG = Optic pathway glioma; PmD = Psychomotor delay; PS = Pulmonary stenosis; RMS = Rhabdomyosarcoma; SF = Spinal form; SS = Short stature (<3rd PCTL; <10th PCTL general population); Thor = Thorax abnormalities (excavatum or carinatum, asymmetric); UH = Umbilical herniation; VS = Vertebral scalloping; XG = Xantogranulomas; n.a. = not available.
Table A4. Unique variants identified in other disease genes: KIT (RefSeq NM_000222.2), LZTR1 (RefSeq NM_006767.3), NF2 (RefSeq NM_000268.3), PPP1CB (RefSeq NM_206876.1), PTEN (RefSeq NM_000314.4), PTEN11 (RefSeq NM_002834.3), SOS1 (RefSeq NM_005633.3), TSC1 (RefSeq NM_000368.4), and TSC2 (RefSeq NM_000548.3).

| Family ID 1 | Gene  | Exon | Type 2 | Genomic | cDNA | Effect | Protein  | ClinVar/HGMD/LOVD ID 3 |
|-------------|-------|------|--------|---------|------|--------|----------|-----------------------|
| 240 (1)     | KIT   | 14   | DEL    | 2027del | 2027del | Frame-shift | Gly676Valfs*4 | New                   |
| 298 (1)     | LZTR1 | 4    | SNV    | 333G>A  | 333G>A  | Missense | Arg118His | LOVD: LZTR1_000051    |
| 323 (1)     | NF2   | 13   | SNV    | 1396C>T | 1396C>T | Nonsense | Arg466*  | ClinVar: 3295         |
| 226 (1)     | 2-6   | DUP  | (114+1_115-1)_(599+1_600-1)dup | n.a. | Intragenic duplication | ? | New                   |
| 263 (1)     | PPP1CB| 3    | SNV    | 146C>G  | 146C>G  | Missense | Pro49Arg | ClinVar: 254648       |
| 258 (2)     | PTEN  | 7    | INS    | 778_779insG | 778_779insG | Frame-shift | Lys260Argfs*38 New |
| 287 (1)     | SNV   | 3    | 235C>A  | 235C>A  | Missense | Glu79Lys | ClinVar: 44605        |
| 242 (1)     | SNV   | 8    | 923A>G  | 923A>G  | Missense | Asn308Ser | ClinVar: 13327        |
| 191 (1)     |      |      |        |         |        |         |          |                       |
| 204 (1)     |      |      |        |         |        |         |          |                       |
| 257 (1)     | PTEN11| 12   | SNV    | 1403C>T | 1403C>T | Missense | Thr468Met | ClinVar: 13331        |
| 272 (1)     |      |      |        |         |        |         |          |                       |
| 236 (1)     |      |      |        |         |        |         |          |                       |
| 301 (1)     |      |      |        |         |        |         |          |                       |
| 270 (1)     | SOS1  | 4    | SNV    | 429G>T  | 429G>T  | Missense | Gly443Ilefs*15 ClinVar: 421669 |
| 187 (2)     |      |      |        |         |        |         |          |                       |
| 189 (2)     |      |      |        |         |        |         |          |                       |
| 265 (1)     | TSC1  | 18   | SNV    | 2293C>T | 2293C>T | Nonsense | His669Asp | ClinVar: 48934        |
| 274 (1)     |      |      |        |         |        |         |          |                       |
| 305 (1)     |      |      |        |         |        |         |          |                       |
| 267 (1)     | TSC2  | 1-22 | DEL    | c.-30+1_29-1)_(-1716+1_1717-1)del | n.a. | Intragenic deletion | ? | New                   |
| 295 (1)     |      |      |        |         |        |         |          |                       |
| 303 (1)     |      |      |        |         |        |         |          |                       |
| 248 (1)     |      |      |        |         |        |         |          |                       |

1 Number of family members presenting the variant is reported in parentheses; 2 Type of variant: SNV = Single-nucleotide variant, DEL = Deletion, DUP = Duplication, INS = Insertion. 3 ID of annotated variants in ClinVar (www.ncbi.nlm.nih.gov/clinvar), Human Genome Variation Database (HGMD; www.hgmd.cf.ac.uk) and Leiden Open Variation Database (LOVD; databases.lovd.nl/shared Genes).
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