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Reassessment of the NF1 variants of unknown significance found during the 20-year activity of a genetics diagnostic laboratory

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ABSTRACT

The finding of variants of uncertain significance (VUS) in the activity of a diagnostic genetic laboratory is a common issue, which is however provisional and needs to be periodically re-evaluated, due to the continuous advancements in our knowledge of the genetic diseases. Neurofibromatosis type 1, caused by the occurrence of heterozygous pathogenic *NF1* variants, is a good model for studying the evolution of VUS, due to the widespread use of genetic testing for the disease, the constant enrichment of the international databases with *NF1* variants and the full adult penetrance of the disease, which makes genotyping the parents a crucial step in the diagnostic workflow. The present study retrospectively reviewed and reinterpreted the genetic test results of *NF1* in a diagnostic genetic laboratory in the period from January 1, 2000 to December 31, 2020. All the VUS were reinterpreted using the 2015 consensus standards and guidelines for the interpretation. Out of 589 *NF1* genetic tests which were performed in the period, a total of 85 VUS were found and reinterpreted in 72 cases (84.7%): 21 (29.2%) were reclassified as benign/likely benign, whereas 51 (70.8%) were recoded as pathogenic/likely pathogenic with a significant trend distribution (Chi square test for trend $p = 0.005$). Synonymous VUS have mainly been reclassified as class 1 and 2 (7/8, 87.5%), whereas missense variants have been attributed to class 4 and 5 in 38 out of the 58 cases (65.5%). These findings underline an improvement in the classification of variants over time, suggesting that a reinterpretation of the genetic tests should be routinely performed to support the physicians in the clinical diagnosis of genetic diseases.

1. Introduction

As genetic testing has become part of the standard assessment of patients with a suspected genetic basis of their disorders, an increasing number of tests is performed worldwide and more attention is given to a major drawback of this routine, which is represented by the uncertainty in the interpretation when variants of unknown significance (VUS) are found (Rosenthal et al., 2017). Finding a VUS may increase the anxiety in patients and their families (Hoffman-Andrews, 2018) and may leave the physician in the clinical dilemma if, for example, to actively adopt the screening strategies for the disease or to passively wait and see. The result, in both cases, is severely flawed by the risk of mismanagement of the disease, by possibly suggesting unwarranted recommendations on one side or, on the contrary, by delaying due clinical decisions (Macklin et al., 2019).

A consensus has been attempted through the years (Richards et al., 2008), culminating in 2015 with the standardization of the VUS interpretation, consisting in the alignment of the clinical genetics laboratories with the guidelines of the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology

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(AMP) (Richards et al., 2015), based on a detailed framework of scoring rules of different types of information, including segregation, computational and functional data.

In view of the progressive update of the public databases, VUS can be reclassified to a different level of pathogenicity, suggesting that an effective strategy would be to periodically re-analyse the laboratory records, as proposed by the ACMG/AMP guidelines (Richards et al., 2015). The recommendation, however, has not yet been translated into clinical procedures in routine diagnostic laboratories about how and when the re-analysis should be done and how patients should be informed. In this context, while the ambiguity of the VUS is becoming increasingly prevalent as a result of the use of more comprehensive diagnostic gene panels or whole exome or genome analysis, other gene tests are becoming more robust, due to the standardization of the interpretation criteria (Richards et al., 2015) and to the enrichment of the population databases, such as ClinVar (Landrum et al., 2014). Neurofibromatosis type I genetic testing belongs to this latter, being frequently tested (the disease has an annual incidence of 1 in 2000–2500 – Uusitalo et al., 2015; Poyhonen et al., 2000; Evans et al., 2010) and relying for the diagnosis on a solid combination of clinical signs and symptoms defined by the National Institute of Health (NIH) Consensus Development Conference in 1988 (Neurofibromatosis, 1988) and recently up-to-dated (Legius et al., 2021).

To evaluate the extent to which a change in the clinical interpretation of variants has occurred through the years in a clinical laboratory, we report the results of a re-analysis of all the VUS found at the Laboratory of Medical Genetics of the University Hospital of Parma in the years 2000–2020 during the *NF1* gene testing, whose clinical interpretation was re-evaluated in the light of the last ACMG/AMP criteria.

2. Methods

A total of 85 VUS were registered among of the 589 *NF1* gene tests performed at the clinical diagnostic Laboratory of Medical Genetics of the University Hospital of Parma between January 2000 and December 2020. Genetic tests were performed by Denaturing High Performance Liquid Chromatography (DHPLC, Transgenomic, US) in the period 2000–2016, whereas, starting from January 2017, Next Generation Sequencing (NGS) protocols have been adopted with an Illumina MiSeq system and Ampliseq Custom Amplicon method, as previously described (Bonatti et al., 2017). Moreover, starting from 2005, copy number variation analysis has been applied to the negative patients using Multiplex Ligation-dependent Probe Amplification (P081–P082 MLPA, MRC Holland) for the detection of deletions or duplications spanning one or more exons, as previously described (Bonatti et al., 2017). Each sequence variant was reported according to the Human Genome Variation Society recommendations (den Dunnen et al., 2016) using the NM_000267.3 transcript sequences. From 2000 to 2016, variant interpretation of *NF1* sequence was based on family segregation, literature, Google and public databases such as ClinVar (Landrum et al., 2014) and LOVD (Fokkema et al., 2011); since 2017, variant classification followed also the ACMG/AMP 2015 guidelines, whereas the use of the VarSome Engine was implemented from 2018 (Kopanos et al., 2018).

The 85 *NF1* selected VUS were reviewed and reclassified (Tables 1 and 2) according to their familial segregation, where available, and to the ACMG/AMP guidelines (Richards et al., 2015). The VUS were independently reinterpreted by three investigators (DM, VB and CM): in the case of a discordant evaluation, a final consensus was obtained. At the end, the initial VUS interpretation and the new classification based on the ACMG/AMP criteria were compared. Statistical analysis was performed with GraphPad Prism software version 9.0.0. Categorical variables, given as percentage of group totals, were analysed through Chi-square with Yates correction and by Chi-square test for trend. A p-value less than 0.05 (two-sided) was considered statistically significant. All the variants have been submitted to ClinVar (Accession numbers: SCV002567751 to SCV002567811). The study has been approved by the local Ethics Committee (603/2019).

3. Results

Out of the 589 *NF1* analyses during the last 20 years of clinical genetic testing, 377 (64%) were carried out on patients fulfilling the clinical criteria for Neurofibromatosis type I (Neurofibromatosis, 1988), for whom a molecular diagnosis was achieved in 317 cases (84% of those satisfying the clinical criteria). In addition, the laboratory records showed the presence of 85 VUS in the same number of unrelated probands (58 missense, 8 synonymous, 1 in-frame deletion, and 18 intronic/near-splice variants) (Tables 1 and 2). After genotyping the parents and after the re-analysis with the assignment to one of the ACMG/AMP pathogenicity classes, 72 (84.7%) of them changed pathogenicity class ($p < 0.001$, Tables 1 and 2): 9/85 (10.6%) were reclassified as benign, 12/85 (14.1%) as likely benign, 39/85 (45.9%) as likely pathogenic, 12/85 (14.1%) as pathogenic (Table 1), resulting in a statistically significant change in the frequency distribution of the variants compared to the original classification ($p < 0.001$). When considering the 52/85 VUS cases fitting the clinical criteria (Neurofibromatosis, 1988) (Table 2), the re-evaluation process led to the attribution of pathogenicity in 40 of them, which resulted in a detection rate of 76.9% of those originally classified as VUS and in an increase of 10.6% of positive tests on the total number of cases fitting the clinical NF1 criteria (40/377), raising the final detection rate of the analysis up to 94.7% (357/377). After sub-dividing according to the type of variant, a statistically significant change ($p < 0.001$) was found for the synonymous (8 variants, 9.4% of the total) and for the missense (58, 68.2% of the total) variants. All synonymous variants have been re-interpreted: in 7/8 cases (87.5%) they were reclassified as benign or likely benign, with a significant distribution trend toward classes 1 and 2 ($p = 0.012$). As far as missense *NF1* VUS are concerned, 46/58 (79.3%) changed class and, of those, 38 VUS became likely pathogenic or pathogenic (65.5%). Distribution trend analysis for missense variants showed a highly

Table 1
Results of the re-analysis of the VUS according to the type of variant.

Type of variant	Unchanged N/T (%)	Reclassified N/T (%)	Chi-square for trend	P-value for trend	Reclassified-1	Reclassified-2	Unchanged	Reclassified-4	Reclassified-5
Synonymous	0/8 (0%)	8/8 (100%)	**6.3**	**0.012**	3	4	0	1	0
Missense	12/58 (20.7%)	46/58 (79.3%)	**11.0**	**< 0.001**	3	5	12	32	6
Nearsplice	1/18 (5.6%)	17/18 (94.4%)	2.2	0.136	3	3	1	6	5
In-frame in/del	0/1 (0%)	1/1 (100%)	NA	NA	0	0	0	0	1
Total VUS	13/85 (15.3%)	72/85 (84.7%)	**8.1**	**0.005**	9	12	13	39	12

N: Variant number.
T: Total number of variants.
NA: Not available.
VUS: Variant of unknown significance.
In Bold significant p-value.

Table 2
List of the NF1 gene VUS under study.

NF1 VARIANT	AMINOACID CHANGE	TYPE OF VARIANT	gnomAD frequency	ClinVar clinical significance	Transcript analysis[b]	Family segregation	Fullfillment of clinical criteria for diagnosis	ACMG/AMP CRITERIA	FINAL PATHOGENICITY CLASS
NM_000267.3:c.107C > G	p.Thr36Ser	Missense	0.00015	Conflicting interpretations: Uncertain significance (1); Benign (1); Likely benign (5)	NA	Inherited by the unaffected mother	NO	BS1, BS2, BS4	1
NM_000267.3:c.1994C > T	p.Ser665Phe	Missense	0.00188	Conflicting interpretations: Uncertain significance (1); Benign (11); Likely benign (5)	NA	NA	NO	BS1, BS2	1
NM_000267.3:c.7532C > T	p.Ala2511Val	Missense	0.00029	Benign/Likely benign	NA	NA	NA	BS1, BS2, BP2	1
NM_000267.3:c.60 + 16C > A	NA	Near-Splice	0.00030	Benign	NA	NA	NA	BS1, BS2, BP2, BP4	1
NM_000267.3:c.654 + 28A > G	NA	Near-Splice	0.00018	Benign/Likely benign	NA	NA	NO	BS1, BS2, BP4, BP6	1
NM_000267.3:c.6999 + 210_6999 + 211insC	NA	Near-Splice	0.00145	Benign[a]	NA	Lack of co-segregation in other affected family members	YES	BS1, BS2, BS4, BP2	1
NM_000267.3:c.4819T > C	p.Leu1607 =	Synonymous	0.01949	Benign	NA	NA	NA	BA1, BP4, BP6, BP7	1
NM_000267.3:c.5730T > C	p.Ile 1910 =	Synonymous	0.00002	Benign/Likely benign	NA	Lack of co-segregation in other affected family members	YES	BS2, BS4, BP4, BP6, BP7	1
NM_000267.3:c.8436T > C	p.Asn2812 =	Synonymous	0.00006	Benign/Likely benign	NA	Inherited by the unaffected mother	NO	BS1, BS2, BS4, BP4, BP6, BP7	1
NM_000267.3:c.655-37A > C	NA	Near-Splice	0.00019	Likely benign[a]	NA	NA	NA	BS2, BP4	2
NM_000267.3:c.4111-9_4111-7delTGT	NA	Near-Splice	0.00035	Benign/Likely benign	NA	NA	NA	BS1, BS2, PP3, PM2	2
NM_000267.3:c.1889T > A	p.Val630Glu	Missense	Absent	Likely benign[a]	NA	Inherited by the unaffected mother	YES	BS4, BP2, PM2	2
NM_000267.3:c.2322T > C	p.Thr774 =	Synonymous	Absent	Likely benign	NA	Inherited by the unaffected father	NO	BS4, BP4, BP6, BP7	2
NM_000267.3:c.6978 > C	p.Ser2326 =	Synonymous	0.000004	Likely benign[a]	NA	NA	NA	BP4, BP7	2
NM_000267.3:c.7353T > C	p.Pro2451 =	Synonymous	Absent	Likely benign	NA	Inherited by the unaffected father	NO	BS4, BP4, BP6, BP7	2
NM_000267.3:c.7584A > G	p.Ser2528 =	Synonymous	0.00001	Likely benign	NA	NA	YES	BP2, BP4, BP6, BP7	2
NM_000267.3:c.4463G > A	p.Arg1488His	Missense	0.00006	Conflicting interpretations: Uncertain significance (4); Likely benign (4)	NA	Inherited by the unaffected mother	NO	BS2, BS4, BP4	2
NM_000267.3:c.584A > G	p.Lys195Arg	Missense	0.00001	Conflicting interpretations: Uncertain significance (6); Likely benign (1)	NA	Inherited by the unaffected mother	NO	BS4, PM2, BP4	2
NM_000267.3:c.7868C > A	p.Ala2623Glu	Missense	Absent	Likely benign[a]	NA	Inherited by the unaffected mother	YES	BS4, BP2, PM2, PP3	2
NM_000267.3:c.3974 + 141T > C	NA	Near-Splice	0.00010	Likely benign[a]	NA	Inherited by the unaffected mother	NO	BS4, BP4	2
NM_000267.3:c.3883A > G	p.Thr1295Ala	Missense	0.00004	Conflicting interpretations: Uncertain significance (10); Likely benign (5)	NA	NA	YES	BS2, BP2	2
NM_000267.3:c.1004A > G	p.Asn335Ser	Missense	0.00001	Uncertain significance	NA	NA	NA	PM2	3
NM_000267.3:c.1460G > A	p.Arg487Lys	Missense	Absent	Uncertain significance[a]	NA	NA	YES	PM2, BP4	3
NM_000267.3:c.1477C > G	p.Leu493Val	Missense	Absent	Uncertain significance[a]	NA	NA	YES	PM2, PP3, PP4, BP2	3

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Table 2 (*continued*)

NF1 VARIANT	AMINOACID CHANGE	TYPE OF VARIANT	gnomAD frequency	ClinVar clinical significance	Transcript analysis[b]	Family segregation	Fullfillment of clinical criteria for diagnosis	ACMG/AMP CRITERIA	FINAL PATHOGENICITY CLASS
NM_000267.3:c.3394C > T	p.Arg1132Cys	Missense	0.00001	Uncertain significance	NA	NA	YES	PM2, PP3, PP4, BP2	3
NM_000267.3:c.3436G > A	p.Val1146Ile	Missense	0.00015	Conflicting interpretations Uncertain significance (13); Likely benign (4)	NA	NA	YES	BP4, PM1, PP4	3
NM_000267.3:c.4319T > C	p.Met1440Thr	Missense	0.000008	Uncertain significance	NA	NA	NO	PM2, PM5, PP3	3
NM_000267.3:c.548T > A	p.Ile183Asn	Missense	Absent	Uncertain significance[a]	NA	NA	NO	PM2, PP3	3
NM_000267.3:c.6361A > C	p.Ser2121Arg	Missense	Absent	Uncertain significance	NA	NA	NO	PM2, PP3	3
NM_000267.3:c.7178A > C	p.His2393Pro	Missense	Absent	Uncertain significance	NA	NA	NO	PM2	3
NM_000267.3:c.7828A > G	p.Thr2610Ala	Missense	0.00001	Uncertain significance	NA	NA	YES	PM1, PM2, PP5	3
NM_000267.3:c.7847G > A	p.Arg2616Gln	Missense	0.00003	Conflicting interpretations: Uncertain significance (6); Likely benign (3)	NA	NA	NA	BS2	3
NM_000267.3:c.6084G > C	p.Lys2028Asn	Missense	Absent	Uncertain significance	NA	NA	NO	PM2, PP3	3
NM_000267.3:c.289-22A > C	NA	Near-Splice	Absent	Uncertain significance[a]	NA	NA	YES	BP2, BP4, PM2	3
NM_000267.3:c.1235A > G	p.Asn412Ser	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	r.1235 A > G,1231_1260del, p.Asn412Ser, Val411_Asn420del	De novo	YES	PS2, PM2, PP4	4
NM_000267.3:c.667T > C	p.Trp223Arg	Missense	Absent	Pathogenic/Likely pathogenic	NA	NA	YES	PS1, PM2, PP3, PP4, PP5	4
NM_000267.3:c.2851G > T	p.Val951Phe	Missense	Absent	Likely pathogenic[a]	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.2870A > T	p.Asn957Ile	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.3112A > G	p.Arg1038Gly	Missense	Absent	Likely pathogenic[a]	r.3112 A > G, p.Arg1038Gly	Inherited by the affected father	YES	PM2, PP1, PP3, PP4	4
NM_000267.3:c.3437T > A	p.Val1146Asp	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (3)	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.3589G > A	p.Ala1197Thr	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	NA	De novo	NO	PS2, PM2, PP3	4
NM_000267.3:c.3651T > G	p.Asp1217Glu	Missense	Absent	Likely pathogenic[a]	NA	Inherited by the affected mother	NO	PM1, PM2, PP1, PP3	4
NM_000267.3:c.4099T > C	p.Cys1367Arg	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.4852T > G	p.Tyr1618Asp	Missense	Absent	Likely pathogenic[a]	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.6055T > C	p.Ser2019Pro	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.6962T > G	p.Leu2321Arg	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	NA	De novo	NA	PS2, PM2, PP3	4
NM_000267.3:c.2410-13A > G	NA	Near-Splice	Absent	Pathogenic/Likely pathogenic	NA	Cosegregation in multiple affected family members	YES	PS1, PM2, PP1, PP4, PP5	4

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Table 2 (*continued*)

NF1 VARIANT	AMINOACID CHANGE	TYPE OF VARIANT	gnomAD frequency	ClinVar clinical significance	Transcript analysis[b]	Family segregation	Fullfillment of clinical criteria for diagnosis	ACMG/AMP CRITERIA	FINAL PATHOGENICITY CLASS
NM_000267.3:c.1585C > T	p.Leu529Phe	Missense	Absent	Likely pathogenic	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.1733T > C	p.Leu578Pro	Missense	Absent	Conflicting interpretations: Likely pathogenic (3); Uncertain significance (1)	NA	NA	YES	PM2, PM5, PP3, PP4	4
NM_000267.3:c.2392A > C	p.Lys798Gln	Missense	Absent	Likely pathogenic*	NA	NA	YES	PM2, PP3, PP4, PP5	4
NM_000267.3:c.3104T > C	p.Met1035Thr	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (4)	r.3104 T > C, pMet1035Thr	NA	YES	PM2, PM5, PP3, PP4	4
NM_000267.3:c.3479G > A	p.Gly1160Asp	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	NA	Inherited by the affected mother	YES	PM1, PM2, PP1, PP4, PP3	4
NM_000267.3:c.3706T > C	p.Trp1236Arg	Missense	Absent	Conflicting interpretations: Likely pathogenic (2); Uncertain significance (1)	NA	Cosegregation in multiple affected family members	NA	PS1, PM2, PP1, PP3, PP5	4
NM_000267.3:c.3834C > G	p.Asn1278Lys	Missense	Absent	Pathogenic/Likely pathogenic	NA	De novo	YES	PM1, PM2, PM6, PP3, PP4, PP5	4
NM_000267.3: c.4109_4110delAGinsCC	p.Gln1370Pro	Missense	Absent	Likely pathogenic[a]	NA	De novo	YES	PM1, PM2, PM6, PP3, PP4	4
NM_000267.3:c.4180A > T	p.Asn1394Tyr	Missense	Absent	Likely pathogenic	NA	De novo	YES	PM1, PM2, PM6, PP3, PP4, PP5	4
NM_000267.3:c.4267A > C	p.Lys1423Gln	Missense	Absent	Pathogenic/Likely pathogenic	r.4267 A > C, p.Lys1423Gln	NA	YES	PM1, PM2, PP3, PP4, PP5	4
NM_000267.3:c.4294G > A	p.Val1432Ile	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (5)	NA	NA	NA	PM1, PM2, PM5, PP3	4
NM_000267.3:c.4340T > G	p.Val1447Gly	Missense	Absent	Likely pathogenic[a]	NA	NA	YES	PM1, PM2, PP3, PP4, PP5	4
NM_000267.3:c.4469 T > G	p.Leu1490Arg	Missense	Absent	Likely pathogenic	NA	NA	YES	PM2, PM5, PP3, PP4	4
NM_000267.3:c.4481 A > G	p.Gln1494Arg	Missense	Absent	Likely pathogenic	NA	NA	YES	PS1, PM2, PP3, PP4, PP5	4
NM_000267.3:c.5413C > G	p.His1805Asp	Missense	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (1)	NA	NA	NA	PS1, PM2, PM5, PP3	4
NM_000267.3:c.5960 A > C	p.Asp1987Ala	Missense	Absent	Likely pathogenic[a]	NA	NA	YES	PM2, PM5, PP3, PP4	4
NM_000267.3:c.6622 T > G	p.Trp2208Gly	Missense	Absent	Likely pathogenic[a]	NA	NA	YES	PM2, PM5, PP3, PP4	4
NM_000267.3:c.6950 T > G	p.Leu2317Arg	Missense	Absent	Likely pathogenic[a]	NA	NA	YES	PM2, PM5, PP3, PP4	4
NM_000267.3:c.7118 T > G	p.Leu2373Arg	Missense	Absent	Likely pathogenic[a]	NA	Inherited by the affected father	NA	PM2, PM5, PP1, PP3	4
NM_000267.3:c.7126G > A	p.Gly2376Arg	Missense	Absent	Pathogenic/Likely pathogenic	NA	De novo	YES	PS1, PM2, PM6, PP3, PP4, PP5	4
NM_000267.3:c.1062+3 A > G	NA	Near-Splice	Absent	Pathogenic/Likely pathogenic	r.889_1062del, p. Lys297_Lys354del	De novo	YES	PM2, PM6, PP3, PP4, PP5	4
NM_000267.3:c.288+4 A > G	NA	Near-Splice	Absent	Conflicting interpretations: Pathogenic (1); Likely	NA	Inherited by the affected father	YES	PS1, PM2, PP1, PP3, PP4	4

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Table 2 (*continued*)

NF1 VARIANT	AMINOACID CHANGE	TYPE OF VARIANT	gnomAD frequency	ClinVar clinical significance	Transcript analysis[b]	Family segregation	Fullfillment of clinical criteria for diagnosis	ACMG/AMP CRITERIA	FINAL PATHOGENICITY CLASS
				pathogenic (2); Uncertain significance (1)					
NM_000267.3:c.3496+3G > T	NA	Near-Splice	Absent	Likely pathogenic[a]	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.6365-3C > A	NA	Near-Splice	Absent	Conflicting interpretations: Pathogenic (1); Likely pathogenic (1); Uncertain significance (3)	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3:c.7907 + 4_7907+7delAGTA	NA	Near-Splice	Absent	Conflicting interpretations: Likely pathogenic (1); Uncertain significance (3)	r.7807_7907del101, p. Thr2604*	NA	YES	PM2, PM4, PP3, PP4, PP5	4
NM_000267.3:c.4269G > A	p.Lys1423 =	Synonymous	Absent	Conflicting interpretations: Likely pathogenic (3); Uncertain significance (1)	NA	De novo	YES	PM2, PM6, PP3, PP4	4
NM_000267.3: c.3639_3641delAAT	p.Met1215del	Inframe Insertion/ Deletion	Absent	Pathogenic	r.3639_3641del, p. Met1215del	NA	YES	PM1, PM2, PM4, PP3, PP4, PP5	5
NM_000267.3:c.1062G > T	p.Lys354Asn	Missense	Absent	Pathogenic/Likely pathogenic	NA	De novo	NA	PS1, PM2, PM5, PM6, PP3	5
NM_000267.3:c.1466 A > G	p.Tyr489Cys	Missense	0.00001	Pathogenic	r.1466_1572del, p.Tyr489*	Inherited by the affected father	YES	PS1, PM2, PP1, PP4, PP5	5
NM_000267.3:c.4309G > A	p.Glu1437Lys	Missense	Absent	Pathogenic	NA	De novo	YES	PM1, PM2, PM6, PP3, PP4	5
NM_000267.3:c.4402 A > G	p.Ser1468Gly	Missense	Absent	Pathogenic	r.4368_4402del, p. Arg1456fs	De novo	YES	PS1, PM2, PM6, PS3, PP5	5
NM_000267.3:c.479G > C	p.Arg160Thr	Missense	Absent	Pathogenic/Likely pathogenic	NA	NA	YES	PS1, PM2, PM5, PP4	5
NM_000267.3:c.730G > A	p.Glu244Lys	Missense	Absent	Conflicting interpretations: Pathogenic (2); Uncertain significance (1)	r.730G > A,655_730del, p. Glu244Lys, Ala219AsnfsTer37	NA	YES	PS1, PS3, PM2, PP3, PP4	5
NM_000267.3:c.1721+3 A > G	NA	Near-Splice	Absent	Pathogenic	r.1642_1721del, p.Ala548fs	Cosegregation with multiple affected family members	YES	PS1, PM2, PM4, PP1, PP3, PP4, PP5	5
NM_000267.3:c.1642-8 A > G	NA	Near-Splice	Absent	Pathogenic	NA	NA	NA	PS1, PP3, PS3, PM2	5
NM_000267.3:c.2326-6 T > G	NA	Near-Splice	Absent	Pathogenic	NA	NA	NA	PS1, PP3, PM2, PP5	5
NM_000267.3:c.5205+5G > A	NA	Near-Splice	Absent	Pathogenic	r.5152_5205del; p. Phe1719_Val1736del	NA	NA	PS1, PP3, PM2, PP5	5
NM_000267.3:c.5749+5G > A	NA	Near-Splice	Absent	Pathogenic	r.5547_5749del, p. Ser1850fsTer2	Cosegregation with multiple affected family members	YES	PS1, PM2, PP1, PP3, PP4, PP5	5

NA: not available.

[a] Our submission in ClinVar.

[b] Based on data available in the literature and in the databases.

significant shift toward classes 4 and 5 ($p < 0.001$, Table 1). Finally, out of the 18 near-splice VUS (intronic variants falling in the intron/exon boundaries out of the canonical splice sites), 17 (94.4%) changed pathogenicity class, being reclassified as pathogenic or likely pathogenic (11, 61.1%). As expected, global trend distribution for all reinterpreted VUS classes was statistically significant ($p = 0.005$), mainly due to the reclassification of the synonymous and missense variants.

4. Discussion

The role of genetic testing in Neurofibromatosis I has steeply increased in the latest years owing to the technical improvements in the analysis, the growing demand for pre-implantation diagnosis and the new findings of genotype/phenotype correlation (Upadhyaya et al., 2007; Rojnueangnit et al., 2015; Koczkowska et al., 2020; Pasmant et al., 2010; Riva et al., 2022; Bonatti et al., 2017). The importance of the genetic testing in the diagnosis of Neurofibromatosis I has also been acknowledged by the recent revision of the diagnostic criteria (Legius et al., 2021), which has incorporated among the cardinal features of the disease also the presence of a pathogenic variant in the *NF1* gene. Therefore, the *NF1* mutation databases are witnessing an unprecedented enrichment of variants, which are progressively clarifying the mutation landscape of the disease and, as a result, inspiring the recommendation of re-evaluating the VUS as part of the diagnostic process during the follow-up of affected patients (El Mecky et al., 2019). The ACMG/AMP 2015 guidelines suggest the classification of the genetic variants in five categories using various types of evidences, including functional, computational, population and segregation data (Richards et al., 2015). Despite all the efforts for making the evaluation as objective as possible, a residual possibility for a subjective interpretation is left, especially regarding how each type of evidence is applied (Amendola et al., 2016). Moreover, protocols and time-intervals for the review process have not been defined (Richards et al., 2015; Chisholm et al., 2018) and results of the re-classification have seldom been published (El Mecky et al., 2019), especially about how to handle the new available pathogenic information and how to recontact the patients several years after the analysis.

In our study we show that the *NF1* variants which were previously defined as VUS, were significantly reclassified into the other four pathogenicity classes, with a general increase in the detection rate of 10.6% of all the patients satisfying the clinical criteria (Neurofibromatosis, 1988), leading to practical consequences for the patients and their family members (So et al., 2019; Westphal et al., 2020). Among the 85 VUS still present in our databases, 72 were reinterpreted and their clinical significance was modified into “benign” or “likely benign” in 21 (29.2%) and into “pathogenic” or “likely pathogenic” in 51 cases (70.8%). In this process, the genotype of the parents and a careful definition of their disease status (affected/unaffected) have been used as a strong interpretative criterion, owing to the full phenotypic expression of the Neurofibromatosis I in adults (Legius et al., 2021), which represents a peculiar feature of the disease, as opposed to the incomplete penetrance of the majority of the adult-onset hereditary tumor predisposition syndromes (Daly et al., 2017). On the other hand, when sufficient data on the family history/segregation are not available, VUS could not be redefined, thus reaffirming the importance of the variants’ familial segregation, which, together with the functional studies through transcript analysis, stands out as a genuine tool at disposal of the diagnostic laboratories. A special notice must be given about some missense variants, like the p. Arg 1809 (Pinna et al., 2015), which display minimal phenotypes (for example few, discrete CALs, which can easily escape detection) and can finally misguide in the interpretation of the significance of the variant.

In conclusion, our study reports the results of the variants’ reinterpretation in the workflow of a diagnostic laboratory and, more specifically related to the *NF1* testing, the importance of the familial segregation of the variants for the attribution of their clinical significance. This implies, especially in centralized services where hundreds of samples are routinely processed, a continuous effort to establish a direct communication between the laboratory and the referring physician even after the laboratory report has been issued, requiring a novel policy allocating resources for the re-analysis of already existing genetic data.

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The authors declare no conflict of interest.

CRediT authorship contribution statement

Davide Martorana: Formal analysis, Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Valeria Barili:** Formal analysis, Funding acquisition, Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Vera Uliana:** Conceptualization, Investigation. **Enrico Ambrosini:** Investigation. **Matteo Riva:** Investigation. **Erika De Sensi:** Investigation. **Elena Luppi:** Formal analysis, Investigation. **Corinne Messina:** Formal analysis, Investigation. **Edoardo Caleffi:** Supervision. **Francesco Pisani:** Supervision. **Antonio Percesepe:** Formal analysis, Funding acquisition, Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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