

ORIGINAL ARTICLE

Tailoring the American College of Medical Genetics and Genomics and the Association for Molecular Pathology Guidelines for the Interpretation of Sequenced Variants in the *FBN1* Gene for Marfan Syndrome

Proposal for a Disease- and Gene-Specific Guideline

See Editorial by West and Summers

BACKGROUND: The introduction of next-generation sequencing techniques has substantially increased the identification of new genetic variants and hence the necessity of accurate variant interpretation. In 2015, the American College of Medical Genetics and Genomics and the Association for Molecular Pathology proposed new variant interpretation guidelines. Gene-specific characteristics were, however, not considered, sometimes leading to inconsistent variant interpretation.

METHODS: To allow a more uniform interpretation of variants in the *FBN1* (fibrillin-1) gene, causing Marfan syndrome, we tailored these guidelines to this gene and disease. We adapted 15 of the 28 general criteria and classified 713 *FBN1* variants previously identified in our laboratory as causal mutation or variant of uncertain significance according to these adapted guidelines. We then compared the agreement between previous methods and the adapted American College of Medical Genetics and Genomics and the Association for Molecular Pathology criteria.

RESULTS: Agreement between the methods was 86.4% (K-alpha, 0.6). Application of the tailored guidelines resulted in an increased number of variants of uncertain significance (14.5% to 24.2%). Of the 85 variants that were downscaled to likely benign or variant of uncertain significance, 59.7% were missense variants outside a well-established functional site. Available clinical- or segregation data, necessary to further classify these types of variants, were in many cases insufficient to aid the classification.

CONCLUSIONS: Our study shows that classification of variants remains challenging and may change over time. Currently, a higher level of evidence is necessary to classify a variant as pathogenic. Gene-specific guidelines may be useful to allow a more precise and uniform interpretation of the variants to accurately support clinical decision-making.

Laura Muiño-Mosquera, MD
Felke Steijns, MSc
Tjorven Audenaert, MD
Ilse Meerschaut, MD
Anne De Paepe, MD, PhD
Wouter Steyaert, BSc
Sofie Symoens, MSc, PhD
Paul Coucke, MSc, PhD
Bert Callewaert, MD, PhD
Marjolijn Renard, MSc, PhD*
Julie De Backer, MD, PhD*

*Drs Renard and De Backer contributed equally as joint last co-authors.

Key Words: fibrillin-1 ■ genomics
■ genotype ■ Marfan syndrome
■ mutation

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CLINICAL PERSPECTIVE

The breakthrough of next-generation sequencing techniques in the past decade and the ensuing high output of genetic data poses a major challenge in terms of variant interpretation. The guidelines for variant classification of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology have proven to be a very important step forward to a more standardized way of classifying and interpreting new genetic variants. In practice, when we applied the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines to classify variants in the fibrillin-1 gene (*FBN1*), the causal gene for Marfan syndrome, we found that application of these guidelines was challenging because some of the proposed criteria were still open to subjective interpretation, leading to inconsistent variant classification. We, therefore, adapted 15 of the 28 general criteria of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology guidelines to better fit some specific features of the *FBN1* gene and the Marfan syndrome. Subsequently, we used these tailored guidelines to classify 713 variants in *FBN1*. The classification according to the newly adapted guidelines and the former practices agreed in 86.4% of the cases. A higher level of evidence was necessary to classify a variant as (likely) pathogenic according to the new adapted guidelines which resulted in a higher number of variants of uncertain significance. Despite the increased molecular uncertainty, this level of caution is warranted to correctly support clinical decision-making. These tailored guidelines could be useful for laboratories offering *FBN1* gene analysis to make the classification more precise and subsequently allowing a more accurate clinical application.

Next-generation DNA sequencing has markedly advanced mass data generation at a lower cost. As a consequence of easily accessible large gene panel testing or exome/genome analysis, considerable numbers of variants that need correct interpretation are identified. The latter is an arduous process, especially when clinical data required to correlate a genotype with the phenotype are lacking. The significance of identified variants can range from certainly pathogenic to certainly benign, but in many cases, the clinical significance of these variants remains uncertain. Furthermore, variant interpretation can differ between laboratories.¹ For clinical purposes, precise classification and interpre-

tation is, however, essential in the diagnostic, therapeutic, and genetic counseling processes.

In 2015, the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) released new general guidelines to aid the correct classification and interpretation of variants in Mendelian diseases.² First, the guidelines recommend indicating the level of pathogenicity, as pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), likely benign (LB), or benign (B). The terms LP and LB indicate that a certain variant is 90% likely to be pathogenic or benign, respectively. Second, the guidelines provide a methodology to allow for this variant classification. This methodology includes an evaluation of 7 different categories (1) the prevalence of the variant in population databases, (2) genotype–phenotype data, (3) literature and locus-specific databases, (4) computational and predictive data, (5) functional data, (6) cosegregation analysis, and (7) allelic data. Several levels of evidence were defined for each of these categories, that is, supportive (P), moderate (M), severe (S), and very severe (VS) resulting in a total of 28 different criteria which can be evaluated. Subsequently these criteria can be combined according to a set of rules to reach a decision on the level of pathogenicity.²

Although the ACMG/AMP guidelines represent a major step forward to the standardization of variant classification, the practical application of these guidelines remains challenging because some of the proposed criteria are still open to subjective interpretation.^{3–5} To outbalance this problem, several initiatives have been undertaken: (1) different computational programs have been developed to facilitate the use of the ACMG/AMP guidelines. These tools can reduce human errors, enable critical evaluation of pathogenicity, and detect and resolve discordant conclusions.^{5–7} Importantly, these tools lack gene-specific knowledge. (2) Another initiative has been the refinement of the ACMG/AMP guidelines by using 108 detailed specifications and a scoring system to classify variants in different genes more precisely.⁸ This concise approach is complex and in need of validation. (3) Further initiatives such as ClinVar, an archival database for genetic variants, have allowed laboratories to share and compare the classification of a concrete variant and to resolve discordant interpretations.⁹ (4) Another partner initiative to ClinVar, the Clinical Genome Resource (ClinGen),¹⁰ incorporates disease- and gene-specific knowledge to define the clinical relevance of genes and variants. Up to date, very limited data on variant curation is available. Knowledge of disease- and gene-specific characteristics is extremely useful for variant classification and advocate for additional disease- and gene-specific guidelines.¹¹

The *FBN1* (fibrillin-1) gene (*FBN1*, OMIM number 134797) encodes the multidomain protein fibrillin-1

(Figure 1). This protein is composed of 47 EGF (endothelial growth factor)-like domains of which 43 are calcium-binding (cb-EGF), 7 TGF (transforming growth factor)- β -binding (TB) domains, 2 hybrid (Hyb) domains, and the N- and C-terminal domains. Each cb-EGF domain contains 6 cysteines, which are pairwise connected through disulphide bonds. Disruption of these bonds has been shown to render the protein more vulnerable to proteolysis and most likely affect its function.^{12–14} Furthermore, cb-EGF domains contain specific cb-sites ((D/N) X (D/N) (E/Q) X_m (D/N) X_n (Y/F), where m and n are variable number of residues and D, aspartic acid; N, asparagine; E, glutamic acid; Q, glutamine; Y, tyrosine; and F, phenylalanine). These cb-sites confer structural stability to the protein, provide protection against degradation, and control interaction with other components of the extracellular matrix.^{14,15}

Pathogenic variants in *FBN1* cause a range of connective tissue disorders, collectively known as type 1 fibrillinopathies.¹⁶ The most well-known and the focus of our study is Marfan syndrome (MFS, OMIM number 154700, ORPHA number 284963). MFS is an autosomal dominant inherited connective tissue disorder affecting multiple organ systems. Prominently affected are the cardiovascular, ocular, and skeletal systems, although manifestations in the skin, lungs, and dura are also commonly associated. The diagnosis is made based on the revised Ghent criteria.¹⁷ In the index person and in the absence of family history, the combination of aortic root dilatation (ARD) or dissection and ectopia lentis (EL) or the combination of ARD or dissection and a positive systemic score will establish the diagnosis. Molecular analysis of the *FBN1* gene is not strictly necessary for the diagnosis, but the identification of a pathogenic *FBN1* variant is helpful in those patients presenting with partial features of MFS. Moreover, it can also help to distinguish MFS from other related disorders presenting overlapping features such as Loeys–Dietz syndrome

(OMIM numbers 609192 and 610168, ORPHA number 60030), MASS phenotype (Mitral valve, Myopia, Aorta, Skin, Skeletal features [OMIM number 604308, ORPHA number 99715]) or mitral valve prolapse syndrome (OMIM number 157700, ORPHA number 741).¹⁷

This study addresses gene- and protein-specific characteristics in the interpretation of *FBN1* variants. Although some of the proposed refinements may be applicable to other genes, the majority are specific to the *FBN1* gene and to MFS.

MATERIALS AND METHODS

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. The final classification of these variants will be published in the Clinvar database.¹⁸

Sample Selection

All consecutively identified variants from our in-house disease-specific MFS database classified as causal mutation or VUS between June 1990 and December 2016 were used for the study. A total of 713 *FBN1* variants were identified. With increasing knowledge, refinement of genetic techniques and collection of larger numbers of variants, variant classification inherently underwent modifications over this 26-year time period. In the past, classification was mainly based on the assessment of a combination of criteria, including whether or not the variant was located in an important functional domain, presence or absence of the variant in an in-house control population, cosegregation of the variant in the family, interspecies conservation of the affected nucleotide/amino acid, and use of computational prediction programs.¹⁷

These 713 variants were found in a total of 934 probands referred for genetic testing in the context of clinical suspicion for MFS and related disorders, EL syndrome, or familial or isolated thoracic aortic disease. Information on phenotypic features was available in 671 of the 934 probands. Two hundred one underwent a thorough clinical evaluation at our institution. In the remainder, phenotyping was performed by

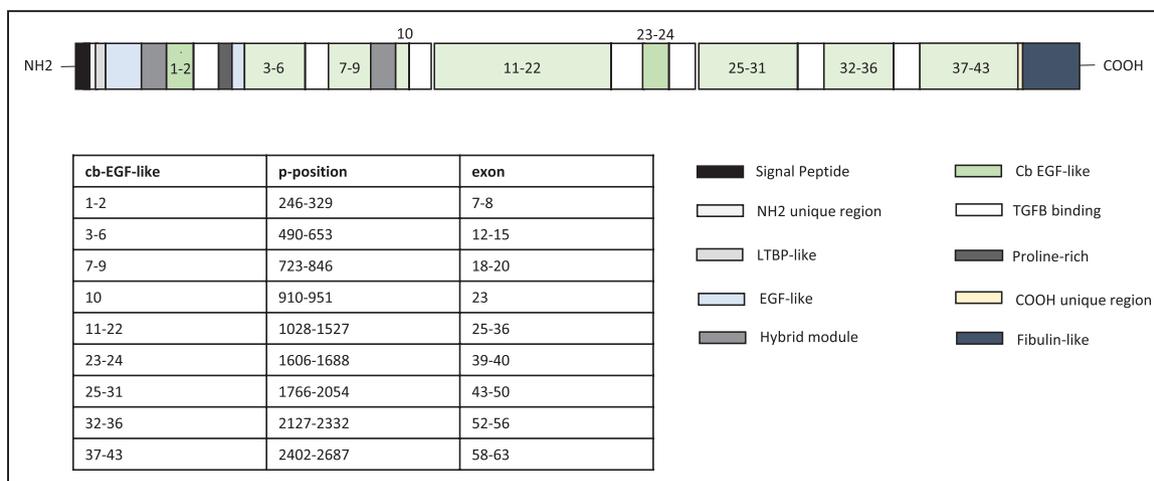


Figure 1. Schematic representation of the *FBN1* (fibrillin-1) protein and the cb-EGF domains.

Cb-EGF indicates calcium-binding-epithelial growth factor domain; COOH, carboxy-terminal; LTBP, latent transforming growth factor β binding protein; NH2, amino-terminal; p-position, amino acid position; and TGFB, transforming growth factor β .

the referring physician, and clinical data were extracted from clinical checklists or request forms.

Variant Detection

FBN1 variants were identified using different mutation-detection methods, including single-stranded conformation polymorphism and conformation sensitive gel electrophoresis, denaturing high-performance liquid chromatography analysis, Sanger sequencing, and next-generation sequencing techniques. Some variants were only detected after using different techniques. All variants were confirmed with Sanger sequencing. Most of the analyses were performed on genomic DNA extracted from peripheral blood leukocytes. A small proportion of the variants was identified in fibroblast-derived complementary DNA. In case of genomic DNA sequencing, all coding exons and flanking intronic sequences (up to 20 base pairs) were analyzed.

Variant Classification

In a working group consisting of molecular laboratory experts (Steyaert, Symoens, Coucke, and Renard) and clinicians (Muiño-Mosquera, Callewaert, De Backer) familiar with MFS and related disorders, we refined 15 of the 28 ACMG/AMP criteria to tailor them to the *FBN1* gene. The *FBN1* gene-specific refinements are summarized in Table 1 and explained below. The genomic sequence used in the classification of the variants was the GRCh37 Human reference assembly (hg19).

Use of Population Databases

Population databases are used to determine the frequency of a certain variant in large populations. Common variants in these databases are more likely considered to be benign, whereas the absence of a variant in these population databases is usually regarded as a pathogenic criterion (PM2). In our study, we considered all *FBN1* variants identified from the dbSNP,¹⁹ Exome Aggregation Consortium,²⁰ Exome Variant Server,²¹ 1000 Genome project,²² and the Genome of the Netherlands²³ databases. Because MFS is a rare disorder with an estimated prevalence between 1.5 to 17.2 per 100 000 individuals,²⁴ the ACMG/AMP allele frequency necessary to be considered as a stand-alone criterion pro benignity (BA1) was reduced from 5% to 1%. Furthermore, an allele frequency above 0.0002 (1:5000) was considered as a strong criterion for benignity (BS1). Finally, case-control studies were only considered to use as a strong criterion (PS4) if >1000 controls were tested.

Use of Phenotypic Data and Alternate Locus Observations

A patient's phenotype or family history highly specific for a monogenic disease is considered supportive evidence for pathogenicity (PP4). In case of MFS, clinical diagnosis can be established either if a patient presents ARD or dissection and EL or ARD or dissection and a positive systemic score. Different studies showed, however, that other syndromic forms of thoracic aortic disease^{17,25} phenotypically overlap with MFS and can present not only ARD but also systemic features similar to MFS. The combination of ARD or dissection and EL seems to be more specific for MFS,¹⁷ and we, therefore, only applied the PP4 criterion when a patient presented these 2 features combined. In addition, when a (likely) pathogenic ((L/P) variant was identified in another known thoracic aortic

disease gene,²⁶ it supported the notion that the *FBN1* variant was possibly benign (BP5).

Use of Literature and (Locus-Specific) Databases

Variant interpretation published in the literature or (locus-specific) databases can be used in support of the classification of a variant of interest (PP5 or BP6). In our study, we used the Human Gene Mutation Database,²⁷ ClinVar,¹⁸ PubMed,²⁸ Leiden Open Variation Database,²⁹ and the Universal Mutation Database for *FBN1*³⁰ databases to search for a specific variant. Only publications providing sufficient clinical or functional evidence were considered for the study. Papers published by our own group were not taken into account.

Use of Computational and Predictive Data

This category includes a variety of computational and predictive criteria, such as the outcome of in silico software tools that predict the impact of a certain variant (BP4, PP3), the predicted effect on the protein's length or expression (BP3, BP7, PM4), and previously reported pathogenic variants affecting the same amino acid or nucleotide (PM5, PS1). To evaluate computational data, we used the following programs: MutationTaster,³¹ SIFT,³² PolyPhen2,³³ Align GVGD,³⁴ and Grantham³⁵ for the missense variants, and Human Splicing Finder,³⁶ GeneSplicer,³⁷ NNSplice,³⁸ and MaxEntScan³⁹ for the splice-site variants. A variant was considered to have a predicted deleterious effect (PP3) or no effect (BP4) on the gene or gene product when at least 3 of the prediction models supported pathogenicity or benignity. If a novel missense variant affected the same nucleotide or amino acid that was previously reported to be (L/P, we considered this criterion as strong (PS1) or moderate (PM5) only when the reported variant was (L/P according to the ACMG/AMP guidelines and if well-established functional studies showed a deleterious effect of that particular variant. Because loss of expression or haploinsufficiency is known as a disease-causing mechanism in the *FBN1* gene, the PSV1 criterion was used for all frameshift and nonsense variants not affecting the final exon 65 and for all splice-site variants in positions ± 1 to 2. Frameshift and nonsense variants affecting exon 65 usually produce a protein which is shorter² and therefore we used for these cases the PM4 criterion.

Use of Functional Data

Robust functional studies showing an effect of the variant on mRNA or protein level are considered strong evidence for pathogenicity (PS3) or benignity (BS3). Cysteine substitutions in the cb-EGF domains of fibrillin-1 are examples of variants affecting well-established and important functional domains with multiple reports of detrimental functional consequences.^{13,14} This type of variant was considered as a strong criterion of pathogenicity. Variants affecting cysteine residues outside cb-EGF domains or residues within the highly conserved cb-sites sequences are less well-established and were regarded as moderate evidence of pathogenicity (PM1).^{14,15}

Use of Segregation Data

The ACMG/AMP guidelines contain several criteria for evaluating segregation. The PM6 can be used if a de novo variant is identified. When paternity and maternity are confirmed, the level of evidence can be increased to a strong criterion (PS2). The guidelines only contemplate one criterion for cosegregation of the variant with the phenotype irrespective to

Table 1. Criteria Used for the Reclassification of the Variants in *FBN1*

Categories	Resource	Criteria of the ACMG/AMP Guidelines	Specifications of the <i>FBN1</i> Classification
Population data	dbSNP	BA1 allele frequency is >5%	BA1 allele frequency is >1%
	ExAC	BS1 allele frequency is greater than expected for the disorder	BS1 allele frequency is >0.0002 (1:5000)
	Exome Variant Server	BS2 observed in a healthy adult individual with full penetrance expected at an early age	
	1000 Genome project	PM2 variant is absent from controls	
	GoNL	PS4 the prevalence of the variant in affected individuals is greater than in controls	PS4 this criterion was used only if at least 1000 controls were included
Genotype and phenotype data		BP5 patient has an alternate molecular basis for the disease*	
		PP4 patient's phenotype or family history is highly specific for a disease with a single genetic origin	PP4 patient has TAD and EL
Literature and (locus-specific) database	HGMD	BP6 variant reported as benign	BP6 and PP5 were used only after critical review of the literature
	ClinVar	PP5 variant reported as pathogenic	
	PubMed		
	LOVD		
	UMD-FBN1		
Computational and predictive data	Missense variants	BP1 missense variant in a gene for which primarily truncating variants are known to cause disease	
	Mutation taster	BP3 inframe deletions/insertions in a repetitive region without a known function.	
	SIFT	BP4 multiple lines of computation evidence suggest no impact on gene or gene product.	BP4 at least 3 lines of computation evidence suggest no impact on <i>FBN1</i> or production of fibrillin-1
	PolyPhen-2	BP7 synonymous variant for which splicing prediction algorithms predict no impact	
	Align GVGD	PP3 multiple lines of computation evidence suggest an impact on gene or gene product.	PP3 at least 3 lines of computation evidence suggest an impact on <i>FBN1</i> or production of fibrillin-1
	Grantham	PM4 protein length changes as a result of inframe deletion or insertion	PM4 protein length changes as a result of inframe change or frameshift or nonsense variant affecting exon 65
	Splice-site variants	PM5 novel missense change at an amino acid residue where a different pathogenic missense change has been seen before	PM5 and PS1 were used only after critical review of the literature and if functional data were available.
	Human Splicer Finder	PS1 same amino acid change previously established as pathogenic regardless of nucleotide change	
	GeneSplicer	PVS1 frameshift, nonsense, canonical $\pm 1-2$ splice-site or exon deletion.	PVS1 frameshift, nonsense, canonical $\pm 1-2$ splice-site or exon deletion (except frameshift or nonsense affecting exon 65)
	NNSplice		
	MaxEntScan		
Functional data	HGMD	BS3 well-established in vitro or in vivo studies show no damaging effect	
	ClinVar	PP2 missense variant in a gene which has a low rate of benign missense variation and which missense variants a well-known disease mechanism	
	PubMed	PM1 located in a mutational hotspot and critical established functional domain	PM1 located in a mutational hotspot and critical established functional domain Cys substitution outside cb-EGF domain Introduction of a new Cys within cb-EGF domain Cb-site substitution in cb-EGF
	LOVD	PS3 well-established in vitro or in vivo studies show damaging effect	PS3 well-established in vitro or in vivo studies show damaging effect Cys substitution within cb-EGF domain
	UMD-FBN1		

(continued)

Table 1. Continued

Categories	Resource	Criteria of the ACMG/AMP Guidelines	Specifications of the <i>FBN1</i> Classification
Segregation data	Segregation analysis	BS4 lack of segregation in affected members of the family	
		PP1 cosegregation with the disease in multiple affected family members in a gene known to cause the disease	PP1 cosegregation with the disease in 1–2 family members PM7 cosegregation with the disease in 3–4 family members PS5 cosegregation with the disease in >4 family members
		PM6 assumed de novo, without confirmation of paternity/maternity	
		PS2 de novo (paternity/maternity confirmed) in a patient with the disease and no family history	
Allelic data		PM3 for recessive disorders detected in <i>trans</i> with a pathogenic variant	
		BP2 observed in <i>trans</i> with a pathogenic variant for a fully penetrant dominant gene or in <i>cis</i> with a pathogenic variant in any inheritance pattern	

Coding system: pathogenic criteria: PVS, very strong; PS, strong; PM, moderate; PP, supportive. Benign criteria: BA, stand-alone; BS, very strong; BM, moderate; PP, supportive. ACMG indicates American College of Medical Genetics; AMP, Association for Molecular Pathology; cb-EGF, calcium-binding-epidermal growth factor; cb-site, calcium-binding site; Cys, cysteine; EL, ectopia lentis; ExAC, Exome Aggregation Consortium; GoNL, Genome of the Netherlands; HGMD, Human Gene Mutation Database; LOVD, Leiden Open Variation Database; and TAD, thoracic aorta disease.

*Other TAD genes considered: TGFBR1/2, SMAD3, ACTA2, TGFB2, and COL3A1.

the number of affected family members carrying the variant (PP1). We adapted the guidelines according to the number of affected family members in whom the variant was present, with 1 to 2 family members affected being a supportive criterion (PP1), 3 to 4 family members being a moderate criterion (PM7), and >4 family members being a strong criterion (PS5).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 24 package (SPSS Inc, Chicago, IL). Unless stated otherwise, continuous variables are expressed as mean and SD and categorical variables as absolute value and percentage. χ^2 and the McNemar tests were used to analyze categorical variables and the K-alpha test to evaluate the agreement between the new classification and the old practices. The Kolmogorov–Smirnov test was used to determine normality. Normal distributed variables were analyzed using the unpaired sample *t* test and the ANOVA and non-normal distributed variables were analyzed using the Mann–Whitney *U* and the Kruskal–Wallis tests. A *P* value of <0.05 was used to define statistical significance (2-sided).

The study was approved by the local Independent Ethics Committee and the Institutional Review Board of our hospital.

RESULTS

A total of 713 *FBN1* variants have been identified in our laboratory in 934 unrelated probands (358 [53.4%] male; mean age, 25.7±16.4 years; 42.8% under the age of 20 years). The majority of these variants were missense variants (n=414, 58.1%), followed by frameshift (n=118, 16.5%), nonsense (n=81, 11.4%), splice-site (n=80, 11.2%), inframe (n=12, 1.7%), and synonymous (n=8, 1.1%) variants. Of the missense vari-

ants, 159 (38.4%) affected a cysteine residue in a cb-EGF domain and 104 (25.1%) affected either a cysteine residue outside a cb-EGF domain or an amino acid in a cb-site. Of the splice-site variants, 43 (53.75%) were in a ±1 or 2 position (Figure 2).

Overall agreement between the new adapted guidelines and the old practices was 86.4% (K-alpha, 0.6). The majority of the variants were reclassified as (L)P (n=526, 73.8%), this was significantly lower in comparison to old practices in which the number of variants classified as causal mutation was 610 (85.5%, *P*<0.001). Consequently, the overall amount of VUS increased from 14.5% to 24.2%. Only 14 variants (2%) were downscaled to LB (Figure 3) and 1 variant was upscaled from VUS to LP. The great majority of the variants in which the new and old interpretation disagreed were downscaled from causal mutation to LB (3) or VUS (82; Table 2). A detailed description of the variants in which there was disagreement can be found in Table I in the [Data Supplement](#). The majority of the downscaled variants from causal mutation to LB or VUS were missense variants outside a cb-EGF domain and not affecting a cysteine residue (n=49, 59.7%), followed by missense variants affecting either a cysteine residue outside a cb-EGF domain or an amino acid in a cb-site (n=13, 15.1%), splice-site variants outside ±1 or 2 position (n=7, 8.1%), frameshift variants in exon 65 (n=7, 8.1%), inframe variants (n=4, 4.7%), synonymous variants (n=3, 3.5%), and nonsense variants in exon 65 (n=2, 2.3%; Table 3). The clinical data needed to correlate phenotype–genotype and segregation analysis was (partly) unavailable in almost half of the cases in which a variant was downscaled (n=35, 40.7% and n=41, 47.7%, respectively).

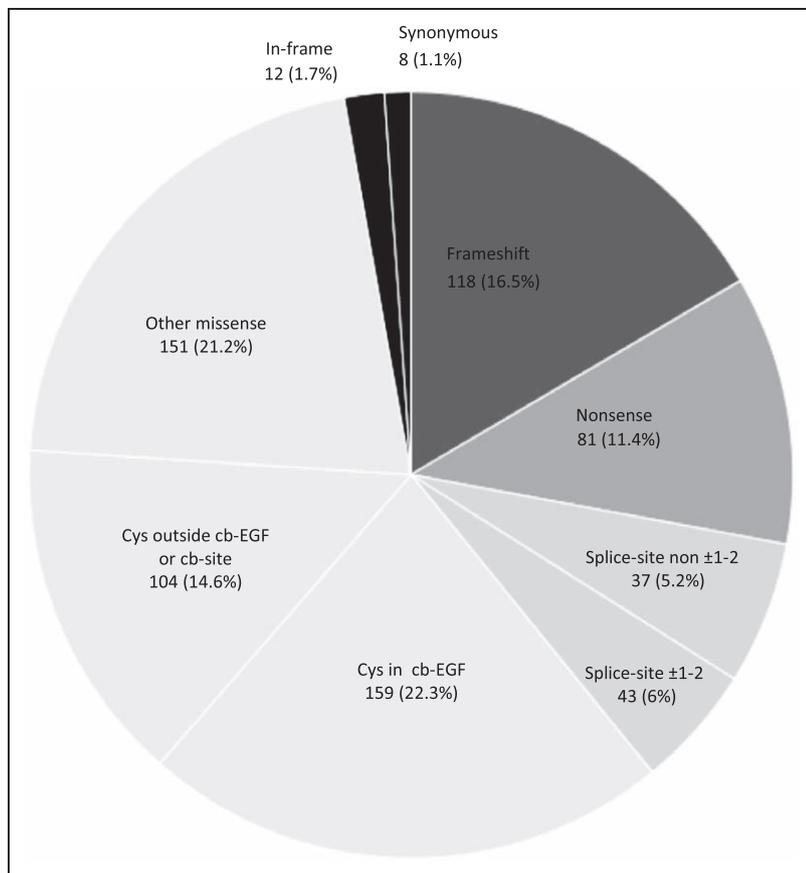


Figure 2. Type of variants found in *FBN1* (fibrillin-1).

cb-EGF indicates calcium-binding-epithelial growth factor domain; cb-site, calcium-binding site; and Cys, cysteine.

All frameshift and nonsense variants not affecting exon 65, all splice-site variants in positions ± 1 to 2 and all missense variants affecting a cysteine residue in a cb-EGF domain were classified as (L)P (Figure 4). Only one of the 8 frameshift and one of the 3 nonsense variants affecting exon 65 could be classified as LP, the rest were classified as VUS. Of the 104 missense variants affecting either a cysteine residue outside a cb-EGF domain or an amino acid in a cb-site, 90 (86.5%) were classified as (L)P. This percentage was much lower for other types of variants: only 32 (21.2%) of the remaining missense variants and 7 (58.3%), 2 (25%) and 6 (16.2%) of the inframe, synonymous, and splice-site variants outside the positions ± 1 to 2, respectively, were (L)P (Figure 4).

Clinical data were available in 671 of the 931 cases. Two hundred sixty-two probands (39%) fulfilled the revised Ghent criteria based on clinical data alone, of whom 160 (61.06%) had ARD or dissection and EL. The remaining had ARD or dissection and a positive systemic score. Of the individuals fulfilling the revised Ghent criteria, 228 (87.1%) had a (L)P *FBN1* variant. This percentage was significantly higher in comparison to the group not fulfilling the Ghent criteria (number of (L)P variants 288, 70.4%, $P < 0.001$). Of the 34 cases, fulfilling the revised Ghent criteria but in whom a VUS or LB variant was identified, 5 individuals carried a frameshift variant in exon 65, 1 individual a nonsense variant in exon 65, and 1 individual a variant affecting a cysteine residue outside a

cb-EGF domain. The 27 other patients had either a missense variant outside a well-established critical domain or a splice-site variant outside positions ± 1 to 2. In 3 of these 27 patients, an additional variant of unknown significance was found: 2 in *FBN1* and 1 in *COL3A1*.

Patients not fulfilling the revised Ghent criteria were significantly younger than those who did (24.42 ± 17.09 versus 27.65 ± 15.23 , $P = 0.011$). This difference was even more apparent when comparing only the groups in which a (L)P variant was found (21.96 ± 16.09 versus 27.35 ± 15.30 , $P < 0.001$) illustrating the age-dependent expression of MFS.

As mentioned in the Materials and Methods section, a distinction in the level of pathogenicity was made based on the amount of family members in which a certain variant in *FBN1* segregated with the phenotype. The moderate (PM7) and the strong (PS5) criteria were used in 20 and in 6 of the classified variants, respectively. In only 3 of these cases, a VUS was upscaled to an LP variant. In 86 cases, the de novo character of the variant was confirmed. In 76 (88.4%) of these 86 cases, the variant was classified as (L)P. In 21 (27.6%) of these 76 cases, de variant was upscaled from VUS to LP.

DISCUSSION

The publication of the ACMG/AMP guidelines was an important step forward in the classification of genetic

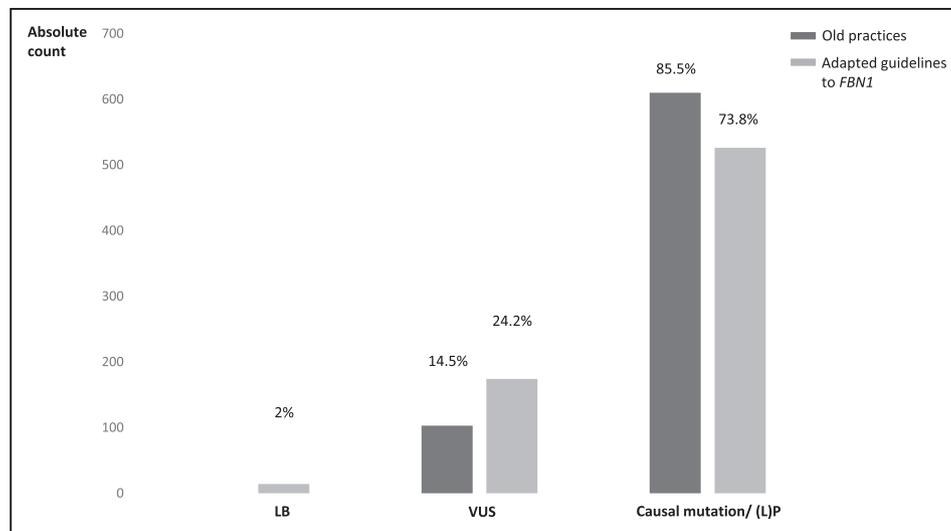


Figure 3. Degree of agreement between the new adapted guidelines to *FBN1* (fibrillin-1) and old practices. LB indicates likely benign; (L)P, (likely) pathogenic; and VUS, variant of uncertain significance.

variants. However, gene-specific criteria were not incorporated in these guidelines allowing for subjective interpretation and considerable interobserver variability in classification outcomes.³

In our study, we tailored some of the ACMG/AMP criteria to specifically suit *FBN1* variant interpretation. When applying the tailored criteria to our cohort, we downscaled 13.9% of the causal mutations to VUS or LB (Table 2) and upscaled only one from VUS to LP. The total percentage of (L)P variants found with the new classification was significantly lower in comparison to the previously used classification methods (73.8% versus 85.5%, $P < 0.001$). This difference can be explained by several factors. First and most important is the increased stringency of the pathogenicity criteria in the new ACMG/AMP guidelines. For example, a missense variant that did not affect an important functional site but segregated in the family and was absent in 400 ethnically matched controls would previously have been considered pathogenic. According to the new guidelines, however, a higher level of evidence is necessary to classify this variant as pathogenic (absence from population databases, clear phenotype–genotype correlation, computational data which predicts pathogenicity or functional studies demonstrating that the variant is pathogenic).

Second, in the new ACMG/AMP guidelines, the variant type strongly influences the final classification. Often, null variants are likely disrupting gene product and function, therefore, counting as a very strong criterion (PVS1) in the variant classification. Similarly, variants affecting a well-established in vitro or in vivo functional site (PS3) are likely to be classified as (L)P. In contrast, other variant types require additional careful phenotyping, segregation analysis, computational tools and, in some cases, functional studies to enable classification as either benign or pathogenic. This phenomenon is clearly reflected in the results of our study. All frameshift and nonsense variants not affecting exon 65, all splice-site variants at positions ± 1 to 2 and all variants affecting a cysteine residue in a cb-EGF domain were classified as (L)P, whereas a variable percentage of (L)P variants was found in the other variant types (Figure 4). Consequently, it is not surprising that the majority of the variants downscaled from causal to VUS or LB were, in fact, missense variants not affecting a well-established functional site (59.7%). The combination of this high percentage of missense variants, insufficient phenotypic or segregation data (40.7% and 47.7% of the downscaled variants, respectively) and stricter guidelines, can partially explain the difference found in our study between the old and the new classification systems.

Table 2. Comparison of the Old and New Classification

		New Classification Based on the ACMG/AMP Guidelines				Total
		LB (%)	VUS (%)	LP (%)	P (%)	
Old classification	VUS (%)	11 (10.7)	91 (88.3)	1 (1)	0	103
	Pathogenic variant (%)	3 (0.5)	82 (13.4)	353 (57.9)	172 (28.2)	610
	Total	14	173	354	172	713

ACMG indicates American College of Medical Genetics; AMP, Association for Molecular Pathology; LB, likely benign; LP, likely pathogenic; P, pathogenic; and VUS, variant of unknown significance.

Table 3. Characteristics of the Downscaled Variants

Characteristics of the Downscaled Variants	
Type of variant, N (%)	Frameshift, 7 (8.1)
	Nonsense, 2 (2.3)
	Cys outside cb-EGF or cb-site, 13 (15.1)
	Other missense, 49 (59.7)
	Splice-site non $\pm 1-2$, 7 (8.1)
	Inframe, 4 (4.7)
	Synonymous, 3 (3.5)
Ghent criteria, N (%)	Yes, 21 (24.4)*
	No, 29 (35.4)
	Insufficient data, 35 (40.7)
Family history, N (%)	Negative, 24 (27.9)
	Proven de novo (no maternity/paternity tested), 4 (4.7)
	Positive, 16 (19.5)
	Insufficient data, 41 (47.7)

cb-EGF indicates calcium-binding-epidermal growth factor; cb-site, calciumbinding site; and Cys, cysteine.

*Seven of these patients presented ectopia lentis and aortic root dilation.

Besides the general increase in stringency of the new ACMG/AMP guidelines, some of the further refinements applied in our study might also have led to the difference in the outcome. Some of the refined criteria, which are open for further debate, are discussed here. First, the allele frequency cutoff for the BS1 criterion was established based on the prevalence of MFS alone, however, a more robust statistical analysis to determine

which frequency of a variant in a reference sample is acceptable might be necessary. A good example is the recent publication of Whiffin et al⁴⁰ in which not only the prevalence of the disease is taken into consideration, but also the estimated contribution of the gene and allele to the disease. Second, the PP4 criterion was used if a patient had ARD or dissection and EL, a combination of features considered more specific for MFS. However, some laboratories might also consider the use of the PP4 criterion if a patient presents a combination of ARD or dissection and a positive systemic score. In our study, the classification of 5 patients would have changed from VUS to LP if the latter consideration had been used. Third, the analysis of data coming from different exome sequencing projects has led to the realization that many variants earlier thought to be pathogenic, are actually present in healthy individuals and are therefore more likely to be considered rare benign variants. Taking this into consideration, in our study the PP5 criterion was used only when sufficient data was provided in the original publication to account the variant as truly pathogenic. Furthermore, because the PM5 and PS1 criteria (variant previously published affecting the same amino acid or the same nucleotide) weigh stronger on the final classification of a variant, these were only used if additional functional evidence of pathogenicity was available. Fourth, although most would agree that variants in *FBN1* affecting a cysteine residue in a cb-EGF domain should be considered as a strong criterion of pathogenicity (PS3), less consensus may exist as to deem the cysteine substitutions in other

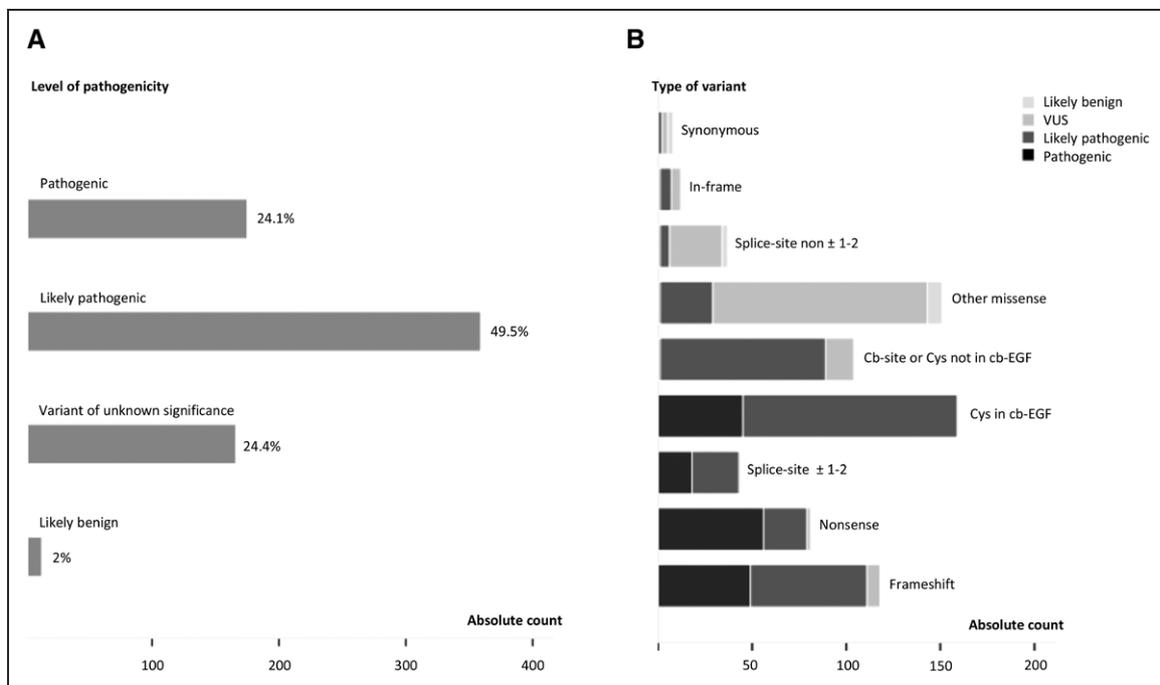


Figure 4. Number of identified variants at each level of pathogenicity.

A, Absolute count and percentage of variants in each level of pathogenicity. **B**, Representation of the level of pathogenicity per type of variant. cb-EGF indicates calcium-binding-epidermal growth factor domain; cb-site, calcium-binding site; Cys, cysteine; and VUS, variant of uncertain significance.

domains or the amino acid substitutions affecting the cb-sites as moderate or strong criterion pro pathogenicity. We decided to make a distinction between these 2 categories based on the number of functional reports. Multiple studies have been published showing that cysteine substitutions in different cb-EGF domains have a deleterious effect on the protein^{13,14,41} and therefore, we decided to generalize this to all cysteine substitutions in these particular domains. In contrast, only 9 studies reported a deleterious effect of missense variants in conserved cb-sites (most affecting a different amino acid of the consensus sequence) and only 2 studies reported on a deleterious effect of cysteine residues in a hybrid or TB domain in fibrillin-1.^{14,15} This was judged insufficient to use as a general rule to support a strong criterion. Fifth, we applied an arbitrary cutoff to the cosegregation rule (PP1), making it a supportive criterion in case 1 or 2 affected family members carried the variant, a moderate criterion when 3 to 4 affected family members carried the variant, and a strong criterion when the number of affected family members carrying the variant was above 4. This rule may, however, be in need of more objective and statistic support. Contrary to the previous refinements, this refinement actually helped classifying 3 variants as LP, whereas otherwise, they would have been VUS.

During the study period, we came to realize that some highly relevant criteria were not included in the current guidelines. First, the frequency with which a particular variant has been reported by independent laboratories could also be considered as a criterion pro pathogenicity. In this case, statistical analysis to determine cutoff values (number of reports) for the strength of the argument would be required. Second, about cosegregation analysis, currently only affected patients carrying the variant or de novo cases count for the classification. Healthy noncarriers and obligate carriers within a family in whom genetic testing is not possible, could, however, also be taken into account when studying cosegregation. Because expression of MFS, especially in young individuals, can be variable, healthy noncarriers should have benefited from careful clinical, echocardiographic, and ophthalmologic examination before being considered for the classification.

In conclusion, variant classification is a dynamic process that can change over time. In this article, we have refined some of the criteria of the ACMG/AMP guidelines to tailor them to the specific characteristics of the *FBN1* gene and its protein to allow a more precise and consistent interpretation of *FBN1* variants. Some of the criteria proposed here might be in need of further debate. Sharing data on functional studies, careful annotation of the variants and appropriate and standard reporting of clinical features could aid variant classification tremendously and subsequently guide clinical decision-making adequately.

ARTICLE INFORMATION

Received November 21, 2017; accepted March 9, 2018.

The Data Supplement is available at <http://circgenetics.ahajournals.org/lookup/suppl/doi:10.1161/CIRCGEN.117.002039/-DC1>.

Correspondence

Laura Muiño-Mosquera, MD, Center for Medical Genetics, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium. E-mail laura.muinomosquera@uzgent.be

Affiliations

Center for Medical Genetics (L.M.-M., F.S., I.M., A.D.P., W.S., S.S., P.C., B.C., M.R., J.D.B.), Division of Pediatric Cardiology (L.M.-M.), and Department of Cardiology (T.A., J.D.B.), Ghent University Hospital, Belgium.

Disclosures

Dr Muiño-Mosquera is supported by a doctoral fellowship from the Special Research Fund (*Bijzonder Onderwijs Fonds*) of the Ghent University. F. Steijns and Dr Meerschaut are supported by a Methusalem grant of the Flemish government and Ghent University to Dr De Paepe. Dr Renard is a postdoctoral researcher of the Research Foundation Flanders. Drs Callewaert and De Backer are senior clinical investigators of the Research Foundation Flanders (*Fonds voor Wetenschappelijk Onderzoek*).

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Tailoring the American College of Medical Genetics and Genomics and the Association for Molecular Pathology Guidelines for the Interpretation of Sequenced Variants in the *FBNI* Gene for Marfan Syndrome: Proposal for a Disease- and Gene-Specific Guideline
Laura Muiño-Mosquera, Felke Steijns, Tjorven Audenaert, Ilse Meerschaut, Anne De Paepe, Wouter Steyaert, Sofie Symoens, Paul Coucke, Bert Callewaert, Marjolijn Renard and Julie De Backer

Circ Genom Precis Med. 2018;11:
doi: 10.1161/CIRCGEN.117.002039

Circulation: Cardiovascular Genetics is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1942-325X. Online ISSN: 1942-3268

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circgenetics.ahajournals.org/content/11/6/e002039>

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SUPPLEMENTAL MATERIAL

Table S1: characteristics of the down- and upscaled variants

c.notation	p.notation	Effect group	FH	Ghent criteria	Criteria	Final Class
c.-35C>T		Splice site non \pm 1-2	Unknown	No	BP6, BP4	2
c.32T>G	p.(Leu11Arg)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.59A>G	p.(Ser648Ser)	Synonymous	Negative	No	PM2, BP4	3
c.206_208del	p.(Pro69_Gly70delinsArg)	In-frame	Positive	Insufficient	PM2, PM4, PP1	3
c.372G>A	p.(Met124Ile)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.863A>G	p.(Asp288Gly)	Other missense	Positive	No	PP3	3
c.939C>G	p.(Cys313Trp)	Cys outside cb-EGF or cb-site	Unknown	No	PM2, PP3, PM1	3
c.1026C>T	p.(Asn342Asn)	Synonymous	Positive	No	BP7	2
c.985A>G	p.(Ile329Val)	Other missense	Positive	No	PM2, BP4, BS4	2
c.986T>C	p.(Ile329Thr)	Other missense	Unknown	No	BS1, BP6, PP3	2
c.1030C>T	p.(Arg344Cys)	Cys outside cb-EGF or cb-site	Negative	No	PP3, PM1	3
c.1073G>A	p.(Cys358Tyr)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PM2, PP3, PM1	3
c.1147G>A	p.(Glu383Lys)	Other missense	Positive	No	PM2, PP5, BP4	3
c.1468G>T	p.(Asp490Tyr)	Other missense	Unknown	Insufficient	PM2, PP5, PP3	3
c.1589A>G	p.(Asp530Gly)	Cys outside cb-EGF or cb-site	Unknown	No	PM2, PP1, PM1	3
c.1766A>G	p.(Asn589Ser)	Other missense	Positive	Insufficient	PM2, PP5, PP3	3
c.1837+5G>A		Splice site non \pm 1-2	Positive	Insufficient	PM2, PP3, PM7	3
c.1837+9T>C		Splice site non \pm 1-2	Unknown	Insufficient	BP6, BP4	2
c.1928T>G	p.(Leu643Arg)	Other missense	Positive	Yes	PM2, PP5, PP3, PP1	3
c.2258G>T	p.(Gly753Val)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.2477G>A	p.(Gly826Asp)	Other missense	Positive	Yes	PM2, PP3, PP1	3
c.2547C>G	p.(Ile849Met)	Other missense	Unknown	No	BP4	2
c.2559C>G	p.(Cys853Trp)	Cys outside cb-EGF or cb-site	Positive	Yes	PM2, PP3, PM1	3
c.2677G>A	p.(Asp893Asn)	Other missense	Positive	Yes	PM2, BP4	3
c.2678-6T>G		Splice site non \pm 1-2	Positive	Yes	PM2, PP3	3
c.2723G>T	p.(Cys908Phe)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PM2, PP3, PM1	3
c.2979C>T	p.(Cys993Cys)	Synonymous	Negative	No	BP6, BP7	2

c.3037G>A	p.(Gly1013Arg)	Other missense	Negative	Insufficient	PM2, PP5, PP3	3
c.3077T>C	p.(Phe1026Ser)	Other missense	Positive	No	BP6, BP4	2
c.3208+5G>A		Splice site non \pm 1-2	Unknown	Insufficient	PM2, PP3	3
c.3212T>G	p.(Ile1071Ser)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PM2, PP3, PM1	3
c.3223C>T	p.(Arg1075Cys)	Cys outside cb-EGF or cb-site	Negative	No	PM2, PP3, PM1	3
c.3232_3236delinsATCTC	p.(Pro1078_Asp1079delinsIleSer)	In-frame	Unknown	Insufficient	PM2, PP5	3
c.3303T>C	p.(Tyr1101Tyr)	Synonymous	Unknown	No	PM2, BP4, BP7	2
c.3382G>C	p.(Val1128Leu)	Other missense	Negative	No	PM2, PP5, BP4	3
c.3509G>A	p.(Arg1170His)	Other missense	Unknown	Insufficient	BS1, BP4	2
c.3554G>A	p.(Gly1185Asp)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.3650G>A	p.(Gly1217Asp)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.3740A>T	p.(Asn1247Ile)	Other missense	Negative	Yes	BP6, PP3	3
c.3815C>A	p.(Ser1272Tyr)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.3937G>A	p.(Gly1313Ser)	Other missense	Unknown	Insufficient	PM2, BP4	3
c.4009G>C	p.(Ala1337Pro)	Other missense	De novo	Insufficient	PM2, PP3, PM6	3
c.4046G>C	p.(Ser1349Thr)	Other missense	Negative	No	PM2, BP4	3
c.4245T>C	p.(Cys1415Cys)	Synonymous	Negative	No	PM2, BP4, BP7	2
c.4389C>A	p.(Asn1463Lys)	Other missense	Positive	Yes	PM2, PP4, PP3	3
c.4460A>C	p.(Asp1487Ala)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PM2, PP3, PM1	3
c.4467T>A	p.(Asn1489Lys)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PM2, PP3, PM1	3
c.4468G>A	p.(Glu1490Lys)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PM2, PP3, PM1	3
c.5076_5078del	p.(Arg1692del)	In-frame	Unknown	Insufficient	PM2, PM4	3
c.5369G>A	p.(Arg1790Gln)	Other missense	Positive	Insufficient	PM2, PP3, PM5, PP1	4
c.5512G>A	p.(Gly1838Ser)	Other missense	Unknown	Insufficient	PM2, PP3, PM5	3
c.5587G>A	p.(Gly1863Arg)	Other missense	Negative	Yes	PM2, PP3	3
c.5722A>C	p.(Thr1908Pro)	Other missense	Negative	Yes	PM2, PP4	3
c.5726T>C	p.(Ile1909Thr)	Other missense	Positive	Yes	PM2, PP4, PP3	3
c.5743C>A	p.(Arg1915Ser)	Other missense	Positive	No	PM2, PP3, PM7	3
c.5743C>T	p.(Arg1915Cys)	Cys outside cb-EGF or cb-site	Negative	No	PM2, PP3, PM1	3
c.5917+3A>C		Splice site non \pm 1-2	Unknown	Insufficient	PM2, PP3	3
c.5921T>C	p.(Ile1974Thr)	Other missense	Positive	No	PP3	3

c.5927A>G	p.(Glu1976Gly)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PM2, PM1	3
c.5983T>C	p.(Ser1995Pro)	Other missense	Negative	Yes	PM2, PP4, PP3	3
c.6073G>T	p.(Ala2025Ser)	Other missense	Unknown	No	BP6, BP4	2
c.6314-15G>A		Splice site non \pm 1-2	Positive	No	BP6, BP4	2
c.6461C>G	p.(Pro2154Arg)	Other missense	Negative	No	PM2, PP3, PP5	3
c.6496G>A	p.(Asp2166Asn)	Cys outside cb-EGF or cb-site	Negative	Insufficient	PM2, PP3, PM1	3
c.6554T>C	p.(Ile2185Thr)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.6659G>A	p.(Arg2220Gln)	Other missense	Negative	No	PP3	3
c.6667A>C	p.(Asn2223His)	Other missense	Positive	Yes	PM2, PP3	3
c.6740A>G	p.(Asp2247Gly)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.6844C>T	p.(Arg2282Trp)	Other missense	Negative	Yes	PM2, PP4, PP5, PP3	3
c.6872-14A>G		Splice site non \pm 1-2	De novo	Yes	PM2, PP4, BP4, PM6	3
c.7003C>T	p.(Arg2335Trp)	Other missense	Unknown	No	PM2, PP3	3
c.7042T>C	p.(Cys2348Arg)	Cys outside cb-EGF or cb-site	Negative	No	PM2, PP3, PM1	3
c.7184G>T	p.(Gly2395Val)	Other missense	Negative	No	PM2, PP3	3
c.7505A>T	p.(Asn2502Ile)	Other missense	Unknown	Insufficient	PM2, PP3	3
c.7699+5G>A		Splice site non \pm 1-2	Unknown	No	PM2, PP3	3
c.7726C>T	p.(Arg2576Cys)	Cys outside cb-EGF or cb-site	Unknown	Insufficient	PP3, PP5, PM1	3
c.776G>A	p.(Gly259Glu)	Other missense	Positive	Yes	PP3, PP1	3
c.7817_7819del	p.Val2606del	In-frame	Unknown	Insufficient	PM2, PM4	3
c.7819+3A>C		Splice site non \pm 1-2	Unknown	Insufficient	PM2, BP4	3
c.7852G>A	p.(Gly2618Arg)	Other missense	Unknown	No	PP3	3
c.7871A>G	p.(Asn2624Ser)	Other missense	Negative	No	PM2, PP3	3
c.7879G>C	p.(Gly2627Arg)	Other missense	Negative	No	PM2, PP5, PP3	3
c.7972C>G	p.(Pro2658Ala)	Other missense	Positive	Yes	PM2, PP3	3
c.7980C>G	p.(Ser2660Arg)	Other missense	Unknown	No	PM2, PP3	3
c.8002G>T	p.(Gly2668Cys)	Cys outside cb-EGF or cb-site	Positive	No	PM2, PP3, PM1	3
c.8177G>A	p.(Arg2726Gln)	Other missense	De novo	No	BS1, PP3, PS3, PM6	3
c.8312dup	p.(Ser2772Glnfs*2)	Frameshift	Unknown	No	PM2, PM4	3
c.8326C>T	p.(Arg2776*)	Nonsense	Unknown	Insufficient	PM2, PP5, PM4	3
c.8363C>T	p.(Thr2788Met)	Other missense	Unknown	Insufficient	BS1, BP6, PP3	2

c.8396delA	p.(Asn2799Metfs*47)	Frameshift	Unknown	Insufficient	PM2, PM4	3
c.8416dup	p.(Ile2806Asnfs*28)	Frameshift	Unknown	Yes	PM2, PM4	3
c.8428del	p.(Glu2810Lysfs*36)	Frameshift	Unknown	Yes	PM2, PM4	3
c.8447A>C	p.(His2816Pro)	Other missense	De novo	No	PM2, PP3, PM6	3
c.8479del	p.(Tyr2827Ilefs*19)	Frameshift	Unknown	Yes	PM2, PM4	3
c.8488C>T	p.(Gln2830*)	Nonsense	Negative	Yes	PM2, PM4	3
c.8516dup	p.(Lys2840Glufs*4)	Frameshift	Negative	Yes	PM2, PM4, PP4	3
c.8525_8529del	p.(Leu2842Profs*7)	Frameshift	Negative	Yes	PM2, PM4	3