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Interpretation and classification of *FBN1* variants associated with Marfan syndrome: consensus recommendations from the Clinical Genome Resource's *FBN1* variant curation expert panel

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Abstract

Background In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) developed standardized variant curation guidelines for Mendelian disorders. Although these guidelines have been widely adopted, they are not gene- or disease-specific. To mitigate classification discrepancies, the Clinical Genome Resource *FBN1* variant curation expert panel (VCEP) was established in 2018 to develop adaptations to the ACMG/AMP criteria for *FBN1* in association with Marfan syndrome.

Methods The specific recommendations were developed through literature review, surveys, online expert panel discussions, and pilot testing of a set of 60 different variants. Consensus among experts was considered reached if at least 75% of the members agreed with a given rule specification. The final set of rules received approval from the ClinGen Sequence Variant Interpretation Working Group.

Results The developed specifications introduce modifications to 14 of the 28 ACMG/AMP evidence criteria and deem 6 criteria non-applicable. Some of these specifications include refining the minor allele frequency thresholds, creating a *FBN1*-specific flowchart for PVS1, defining functional domains of the protein, developing a point-based system of counting probands and instances of de novo occurrences, recommending a points-based method of accounting for family segregation data, and clarifying the applicable functional assays that should be considered. To date, this VCEP has curated 120 variants which have been deposited to ClinVar with the 3-star review status.

Conclusions Establishing specific adaptations for *FBN1* has provided a framework to foster greater classification concordance among clinical laboratories, ultimately improving clinical care for patients with Marfan syndrome.

Keywords Marfan syndrome, *FBN1*, ACMG-AMP guidelines, Variant interpretation, Variant curation

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Background

Pathogenic variation in the fibrillin-1 gene (*FBN1*, MIM *134,797) is associated with Marfan syndrome (MIM #154,700), an autosomal dominant multisystemic connective tissue disorder characterized by a broad and variable phenotypic spectrum involving the cardiovascular, ocular, and skeletal systems. The cardinal characteristics are thoracic aortic aneurysm and dissection (TAAD) and ectopia lentis. Other cardiovascular features, present in a variable number of patients, include mitral valve prolapse, cardiomyopathy, and arrhythmias [1], while additional ocular features such as myopia, astigmatism, and flat cornea may be present [2]. Musculoskeletal manifestations include arachnodactyly, protrusio acetabuli, pectus anomalies, scoliosis, and others, and are usually essential to identify patients with Marfan syndrome.

Fibrillin-1 is a critical structural protein of the extracellular matrix, present in both elastic and non-elastic tissues. Besides its structural role, fibrillin-1 plays a crucial function in mechanosensing and mechanotransduction of environmental changes [3], interacting with various microfibril-associated proteins, growth factors, and cell membrane receptors. Together with its structural significance, fibrillin-1 regulates the bioavailability of TGF-β and therefore direct involvement in processes including inflammation, fibrosis, and matrix metalloproteinase activation results in the characteristic phenotype including the aortic wall weakening [4].

Marfan syndrome is among the more common of the "rare diseases," with an estimated prevalence between 1:5000 and 1:10,000 [5]. Over 3000 different (likely) pathogenic variants spanning all 65 exons of *FBN1* have been reported as causative for Marfan syndrome, a considerable proportion of which have only been reported in a single individual or family [6, 7]. These comprise variants resulting in haploinsufficiency (i.e. nonsense, frameshift, splicing, and gross deletions) and those thought to confer a dominant negative effect, namely by altering cysteine residues or other conserved amino acids in the encoded epidermal growth factor (EGF)-like, calcium-binding EGF-like, TGF-β-binding protein-like, and hybrid domains [8].

The diagnosis of Marfan syndrome can be made clinically without molecular testing via the revised Ghent criteria [9]. These criteria take into consideration the presence of aortic root dilation or history of a dissection, the presence of ectopia lentis, and the systemic score, the latter of which is a combination of mostly skeletal and some non-skeletal features. Genetic testing can dramatically aid in establishing a diagnosis prior to development of the full clinical phenotype. At least two studies have highlighted the underdiagnosis of Marfan syndrome when a genotype-first approach was used in population databases [10, 11]. This is especially important due to the frequent morbidity or mortality from undiagnosed TAAD [12, 13]. Molecular diagnoses also assist clinicians in their medical management decisions by informing the need for earlier clinical monitoring and medical interventions such as prophylactic aortic surgery [14, 15]. Obtaining a molecular diagnosis, especially for patients who do not meet Marfan syndrome clinical diagnostic criteria, allows differentiation from other connective tissue disorders with overlapping phenotypes such as Loeys-Dietz syndrome, Shprintzen-Goldberg syndrome, Meester-Loeys syndrome, and congenital contractural arachnodactyly, as well as the many other genetic etiologies of heritable thoracic aortic disease. Further, and critically, identification of a causative pathogenic variant permits vital cascade screening of potentially affected biological family members and enables more comprehensive counselling about reproductive decision making and family planning options, including prenatal and preimplantation diagnoses [16].

For several years, laboratories have been largely utilizing the same generic framework for interpretation and classification of sequence variants published jointly by the American College of Medical Genetics and Genomics (ACMG) and Association of Molecular Pathology (AMP) [17, 18]. However, significant discrepancies in variant classifications persist due to differences in how laboratories assess evidence and decide to modify and apply the criteria described in the ACMG-AMP guidelines [19, 20]. Mitigating the possibility for confusion, misdiagnosis, or mismanagement caused by inter-laboratory variant classification discrepancies and reducing the frequency with which novel and recurrent variants are classified as of uncertain significance (VUS) are eminently desirable. With these goals, numerous Clinical Genome Resource (ClinGen) variant curation expert panels (VCEPs) have been created to develop gene- or disease-specific modifications to the ACMG-AMP criteria to foster more tailored, accurate, and standardized variant interpretations [21–24].

The high prevalence of Marfan syndrome and the clinical significance of establishing its diagnosis underline the importance of addressing these limitations in interpretation. Further, it emphasizes the potential impact that improvements to the utility that *FBN1* analyses can bring and the need to develop guidance on *FBN1* variant interpretation. To bridge the existing gap in interpretation and classification practices, Muiño-Mosquera et al. developed their own *FBN1*-specific adjustments to the ACMG-AMP criteria within a single institution [8]. Recognizing the importance for broader expertise and consensus, an international group of experts specializing in *FBN1* and Marfan syndrome was then engaged to further

refine these specifications. As a result, the ClinGen's FBN1 VCEP was established aiming to enhance the management of this extensive patient population with two primary objectives. Firstly, the panel aimed to develop consensus recommendations for best practices of *FBN1* variant interpretation, ensuring wider dissemination and implementation among relevant stakeholders. Secondly, leveraging the collective expertise in *FBN1* and Marfan syndrome, the panel sought to provide expert curations of previously identified *FBN1* variants.

Methods

ClinGen FBN1 variant curation expert panel

The *FBN1* VCEP membership represents a multidisciplinary group of medical geneticists and (paediatric) cardiologists, research scientists, molecular genetic diagnostic scientists, and genetic counsellors with a wealth of experience and expertise surrounding *FBN1*, Marfan syndrome, and related connective tissue disorders. Membership currently comprises nine institutions, three of which are designated as the "core" team (Ghent University Hospital, Hôpital Bichat, Mayo Clinic), and spans five countries (Belgium, Canada, France, Japan, USA).

Beginning with the first meeting in November 2018, the VCEP met once every month until the finalization of the rules. Before every meeting, a survey was conducted to gather feedback about the initial proposal for rule specifications from Muiño-Mosquera et al. [8] The survey consisted of multiple-choice questions, with each question providing an option for commentary to include any rationale not covered by the given choices. The survey results informed subsequent discussion and allowed for additional clarification during group discussions. During each meeting, an online voting system was used to reach a consensus. Points of disagreement were discussed until at least 75% agreement was achieved. In most cases, this required reappraisal of the literature or internal data. Once a set of rule specifications were preliminarily agreed upon by the panel, they were piloted by the VCEP to identify possible pitfalls and inconsistencies and thus prompt further refinements. The iterative development of the rule specifications involved guidance from the ClinGen Sequence Variant Interpretation Working Group (SVI) as well as exploitation of other previous VCEP modifications, including reference of the RASopathy VCEP for the PS4 criterion, the Hearing Loss VCEP for the PP1 criterion, and the *MYH7* VCEP for the population frequency cut-off values [21–23]. The ultimate version of the rule specifications was voted and agreed on by a majority of the VCEP members prior to submitting and receiving final approval from the ClinGen SVI. A minimum of 75% agreement among members was deemed necessary to approve a specification, also at this stage. The process of creation and rule development of the *FBN1* VCEP is represented in Fig. 1 and followed the established ClinGen's framework [25].

The ontology used for curation is Marfan syndrome (MONDO:0007947) with autosomal dominant inheritance (HP:0000006). Other diseases associated with *FBN1*, like stiff skin syndrome (MONDO:0008492) or geleophysic dysplasia (MONDO:0013612), were not considered for the specifications. The ClinGen's framework specifically abrogates for creating specifications and performing variant curations for a single gene-disease relationship. There is only one biologically relevant *FBN1* transcript; consequently, all variants are curated according to their annotation on the MANE Select transcript NM_000138.5. All curations are published in the ClinGen Variant Curation Interface under the *FBN1* affiliation and in the ClinVar repository as reviewed by an expert panel (3 stars).

Piloting the rule specifications

Each of the nine constitutive VCEP institutions were asked to contribute 10 *FBN1* variants for possible inclusion in the pilot study that had been identified in patients evaluated at that clinic or tested at that laboratory with a clinical diagnosis or suspicion based on clinical features and/or family history. Notably, as these variants may have been identified at any point in time, testing methodologies at these reputable clinical and research laboratories include various molecular techniques that were standard of practice at that point in time (e.g. denaturing high-performance liquid chromatography [dHPLC], Sanger sequencing and/or next-generation sequencing, with orthogonal variant confirmation when appropriate). Each institution was asked to submit five missense, two frameshift, one nonsense, one splicing, and one in-frame insertion/deletion (indel) variant, including at least two (likely) benign, two VUSs, and two (likely) pathogenic. From that subset of 92 variants, a total of 60 variants were deliberately chosen by the "core" team on the basis of creating a set with challenging interpretations that represent as wide a range of characteristics as possible. This included the variant type (missense, frameshift, nonsense, splicing, in-frame deletion or insertion, synonymous), the amount of available evidence and variety of evidence types (e.g. probands with various degrees of phenotype information available, familial vs. de novo variants, experimental data, variants affecting different putative functional domains/conserved positions), having both recurrent and unique variants, and having each of the five classifications of clinical significance represented in order to comprehensively test the rule modifications.

The six non-core institutions were each assigned 10 variants to interpret and classify using the *FBN1*-specific

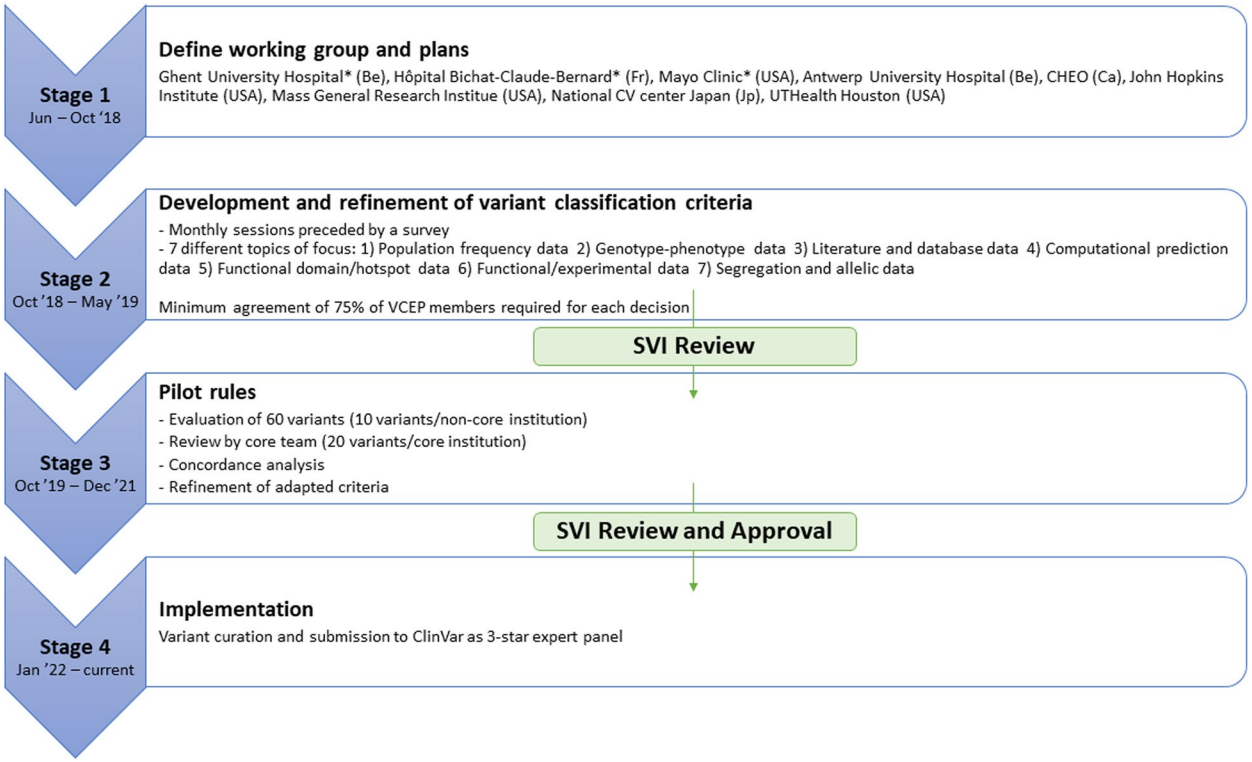

Fig. 1 Overview of the process used for adapting the ACMG/AMP criteria to *FBN1*: the procedure was staged in four phases as defined in the figure. Stages 2 and 3 were followed by evaluation and feedback from the ClinGen Sequence Variant Interpretation Working Group (SVI). Stage 4 consists of an ongoing process of variant curation and submission to ClinVar for public accessibility. *Core members. Abbreviations: Be: Belgium, Fr: France, USA: United States of America, Ca: Canada, Jp: Japan

rules, with institutions' internal case-level data supplementing the publicly available data for interpretation. The variants' original classifications and their reclassifications derived using the VCEP rule specifications were compared. All 60 variants were then reassessed by the three core institutions, with each responsible for evaluating 20 variants. Classification discordance between core and non-core institutions was then calculated, and the reasons for discordance were noted for additional discussion and refinement with the full VCEP and SVI. Variants with discordant classifications were re-interpreted following these discussions and any amendments to the criteria specifications that were made. If internal data at one VCEP institution was key to a variant's discordant classifications, that data was shared so that the interpretation could be repeated with equivalent information available.

Results

Disease-specific adaptations of ACMG-AMP Classification Criteria

Of the 28 individual ACMG-AMP criteria, *FBN1*-specific modifications to utilization and/or strength level were introduced for 14 criteria. Six criteria (PM3, PP5, BS2, BP1, BP3, BP6) were deemed to not be applicable for variants in *FBN1*. Additionally, one change to the ACMG-AMP combining rules was instituted to allow sufficiently rare loss of function variants that satisfy both PVS1 and PM2_Supporting criteria to reach a likely pathogenic classification; this practice accords with recommendations from the ClinGen SVI [26] and has been previously implemented by other VCEPs(23,24). A summary of the specifications can be found in Table 1.

Null variant in a gene where loss-of-function is a known mechanism of disease (PVS1)

Haploinsufficiency is well known as one of the mechanisms of pathogenesis for *FBN1* and Marfan syndrome [27, 28] and the gnomAD probability of being loss-of-function intolerant (pLI) and loss-of-function observed/expected upper bound fraction (LOEUF) scores are 1 and 0.105, respectively; therefore, PVS1 is applicable for all putative loss-of-function variants (i.e. nonsense, frameshift, consensus splice site, large deletions). The VCEP made minor *FBN1*-specific modifications to Abou Tayoun et al.'s PVS1 decision tree developed to guide

Table 1 Overview of the adapted criteria from the ACMG/AMP guidelines to FBN1

Criteria	Modification	Description
Pathogenic very strong		
PVS1	Disease-specific Strength	**- *PVS1:*** nonsense, frameshift, canonical splice site (+/- 1,2) variants, and multi-exon duplications and deletions predicted to undergo NMD **- *PVS1_strong:*** nonsense and frameshift variants affecting the last exon or 55bp of penultimate exon or canonical splice-site- variants and multi-exon duplications and deletions not predicted to undergo NMD **- *PVS1_moderate:*** Initiation codon variant with 1 or more pathogenic variant(s) upstream of closest potential in-frame start codon
Pathogenic strong		
PS1	None	
PS2	ClinGen recommendation Disease-specific	Point-based system recommended by ClinGen SVI (https://clinicalgenome.org/docs/ps2-pm6-recommendation-for-de-novo-ps2-and-pm6-acmg-amp-criteria-version-1.0/) The VCEP defined the phenotypes: **- *Highly specific for disease*:** TAAD + ectopia lentis **- *Consistent with gene but not highly specific*:** TAAD + SS ≥7 **- *Consistent with gene but not highly specific or genetic heterogeneity*:** (isolated) TAAD, isolated ectopia lentis, or in case of child (age <20yrs) systemic score >7
PS3	ClinGen recommendation Disease-specific	ClinGen functional assay recommendations (Brnich et al., 2019) The VCEP defined: - ***Functional studies deemed appropriate****:* cDNA analyses in the presence of NMD inhibitor; *in vitro* engineered system showing abnormal expression, proteolysis, folding, assembly, trafficking, secretion, Ca^{2+} binding, matrix deposition or microfibril fragmentation/catabolism. - ***Functional studies NOT deemed appropriate****:* non-specific altered TGF-beta signalling or histological hallmarks of medial degeneration.
PS4	Disease-specific	1 point: each additional proband fulfilling Ghent criteria or with ectopia lentis. 0.5 points: all other cases with features suggestive of Marfan syndrome **- PS4:** ≥4 points **- PS4_moderate:** 2-3.5 points **- PS4_supportive**: 1-1.5 points
Pathogenic moderate		
PM1	Disease-specific	**- PM1_strong:** Cysteine residues in cbEGF domain **- PM1:** Cysteine residues not in cbEGF domain, Cysteine-creating residues, residues affecting interdomain packaging, conserved residues in consensus calcium-binding sequence *see supplementary file for residues for consideration of PM1
PM2	ClinGen recommendation Disease-specific	Use as **PM2_supporting** ClinGen PM2 recommendations: (https://clinicalgenome.org/site/assets/files/5182/pm2_-_svi_recommendation_-_approved_sept2020.pdf). The VCEP defines: - Threshold <0.0005% - Use highest ethnic population AF
PM3	Disease-specific	N/A for *FBN1*
PM4	None	
PM5	Disease-specific	Don't apply if PM1_strong is applied
PM6	ClinGen recommendation Disease-specific	*See PS2
Pathogenic supportive		
PP1	Disease-specific	**- PP1_strong:** ≥5 additional affected family members **- PP1_moderate:** 4 additional affected family members **- PP1:** 2-3 additional affected family members
PP2	None	
PP3	Disease-specific	- For missense: REVEL score ≥0.750 - For splicing: use GeneSplicer, MaxEntScan, and NNSplice (concordance)
PP4	Disease-specific	- Use if patient fulfils revised Ghent criteria - Can be used if any of the family members have a highly specific phenotype
PP5	ClinGen recommendation	N/A

Table 1 (continued)

Benign stand alone		
BA1	Disease-specific	- Threshold: >0.1% - Use highest ethnic population AF
Benign strong		
BS1	Disease-specific	- Threshold: 0.005 – 0.1% - Use highest ethnic population AF
BS2	Disease-specific	N/A
BS3	None	
BS4	None	
Benign supportive		
BP1	Disease-specific	N/A
BP2	Disease-specific	- *In trans* in ≥ 2 cases with co-occurring pathogenic variants, and phenotype is not more severe than when seen in isolation - *In cis* with a pathogenic variant, if the pathogenic variant has been seen in isolation in a patient with the disease phenotype
BP3	Disease-specific	N/A
BP4	Disease-specific	- For missense: REVEL score ≤0.326 - For splicing: use GeneSplicer, MaxEntScan and NNSplice (concordance)
BP5	None	
BP6	ClinGen recommendation	N/A
BP7	None	

application of PVS1 at varying strengths(29) as shown in Fig. 2.

Assessment of variant minor allele frequency (BA1, BS1, PM2)

The optimal threshold for variant minor allele frequency was calculated according to the recommendations published by Whiffin et al. [30], including the maximum estimated prevalence of Marfan syndrome of 1:5000 individuals (1:10,000 chromosomes) [31], a presumed possible penetrance of TAAD of 80% [32], the contribution of *FBN1* to Marfan syndrome of 90% [9], and an extremely conservative estimate that no pathogenic variant is responsible for more than 40% of cases of Marfan syndrome. PM2 (variant is absent or rare in the general population) was reduced in strength to PM2_Supporting, consistent with a long-standing recommendation from the ClinGen SVI [33]. The threshold for use of PM2_Supporting was established as a minor allele frequency less than 0.0005% (0.000005). For BA1 and BS1, the optimal frequency thresholds were determined to be 0.1% (0.01) and 0.005% (0.00005), respectively. No known *FBN1* pathogenic variants have frequencies exceeding 0.005% in gnomAD; thus, no pathogenic variants would be inappropriately assigned BS1 in support of benignity. The VCEP recommends that for assessment of minor allele frequency data in gnomAD the highest ancestral population frequency should be utilized, with the stipulations that the bottlenecked (i.e. European [Finnish], Ashkenazi Jewish) and "Other" populations should not be utilized, and that any ancestral population being considered should have at least 2000 alleles studied at that position.

Increased variant prevalence in cases versus controls (PS4)

Due to the rarity of *FBN1* pathogenic variants and the inherent inability to perform case–control studies for variants, a points-based system of counting probands with the variant of interest was developed, consistent with the practice employed by many other ClinGen VCEPs associated with conditions with autosomal dominant inheritance [34–37]. Probands reported in internal or public databases or published in the primary literature documented to have ectopia lentis and/or a Marfan syndrome diagnosis based on the revised Ghent criteria [9] are each awarded 1 point. Probands who do not meet the revised Ghent criteria and do not have ectopia lentis (e.g. have isolated TAAD or a systemic score greater than or equal to 7 without TAAD) or whose clinical phenotypes are incompletely described are awarded 0.5 points. The sum of the proband points corresponds to a given PS4 strength: 1–1.5 points is sufficient for PS4_Supporting, 2–3.5 points is sufficient for PS4_Moderate, and 4 or more points is sufficient for PS4. To avoid inappropriate use of the PS4 criterion for variants commonly seen in the general population, PS4 should not be applied at any strength for variants that are frequent enough in gnomAD to apply the BA1 or BS1 or criteria.

Mutational hot spot or well-studied functional domain without benign variation (PM1)

Cysteine residues form disulfide bonds throughout the fibrillin-1 protein and are therefore well-established as critical to the stability and function of the protein [38, 39]. Disulfide bonds in the calcium-binding epidermal growth factor (cbEGF)-like domains are especially crucial, and variants that alter one of these cysteine residues

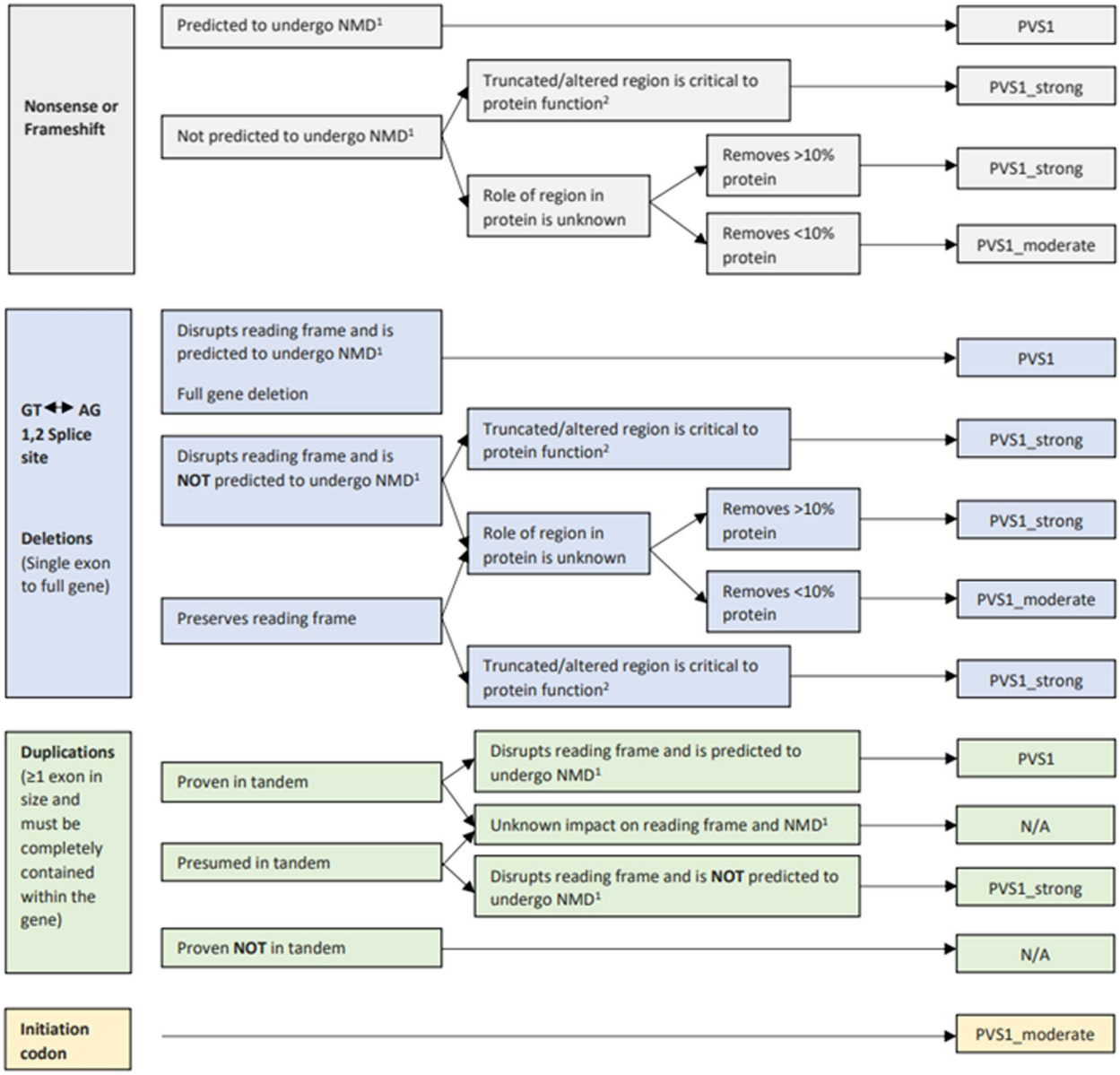

Fig. 2 Flowchart for the adapted PVS1 criterion for null variants: the *FBN1* VCEP made minor modifications to the original PVS1 decision tree developed by ClinGen [29]. The only biological relevant transcript for *FBN1* is NM_000138.5. Additionally, two aspects need to be taken into consideration: (1) NMD (nonsense-mediated mRNA decay) is predicted to occur when a stop codon is integrated in the *FBN1* sequence, except for stop codons in the last exon or the last 50–55 nucleotides of the penultimate exon. (2) A critical region is defined using the same criteria as for the PM1 and PM1_Strong criteria

are among the most prevalent of disease-causing variation in *FBN1* [39]. Therefore, for cysteine-removing variants in any of the 43 cbEGF-like domains, PM1 was increased in strength to PM1_Strong; this encompasses 258 cysteine residues encoded throughout the gene. For cysteine substitutions in the other domains (i.e. EGF-like, TGF-β-binding protein-like, and hybrid) of the gene and for cysteine-creating variants in cbEGF domains, PM1 is applicable at its original moderate strength.

There are numerous other functionally and structurally important non-cysteine residues appropriate for application of PM1 based on their role in either inter-domain packaging (e.g. glycine residues in cbEGF-like domains that are positioned between the second and third cysteines or between the third and fourth cysteines, the latter of which requires the presence of an upstream cbEGF-like domain to be applicable), calcium binding (e.g. variants altering the conserved

residues in the consensus calcium-binding sequence [D]-X-[D/N]-[E/H]-Xm-[D/N]-Xn-[Y/F]), or sites of possible β-hydroxylation (the second [D/N] of the consensus calcium-binding sequence). The VCEP notes that an asparagine-to-serine (N > S) substitution at the second of the aspartic acid or asparagine (D/N) positions might be tolerated based on the frequency of this type of missense variant in gnomAD [40], and PM1 should therefore not be applied in these instances. Including the aforementioned variant types and the cysteine-involved variants outside of the cbEGF-like domains, PM1 can be applied for variants at 375 different amino acid positions. In total, the PM1 criterion can be used at either moderate or strong level for 22.0% (633/2871) of amino acid positions across *FBN1* (Supplementary Table 1). Indeed, the use of PM1 for the above types of non-cysteine variants was a critical factor to the reduction of VUS in favour of likely pathogenic/pathogenic classifications in the pilot study (Fig. 4).

De novo events (PS2, PM6)

The ClinGen SVI has universal recommendations for a points-based application of the PS2 and PM6 criteria that involves consideration of the phenotype of either the patient in question or previously reported probands with de novo inheritance [41]. Application requires an evaluation of the extent to which an individual's phenotype is specific for a certain gene and how much genetic heterogeneity exists for that phenotype. The *FBN1* VCEP recommends utilizing the same points-based framework for utilization of PS2 and PM6 and developed tiers of phenotype specificity and genetic heterogeneity to utilize this SVI-derived system for instances of de novo *FBN1* variation. ClinGen's "Phenotype highly specific for gene" category was defined for *FBN1* as the presence of TAAD and ectopia lentis. The "Phenotype consistent with gene but not highly specific" category was defined as the presence of TAAD and a systemic score greater than or equal to seven. Finally, probands with isolated TAAD, isolated ectopia lentis, or in an individual younger than 20 years of age for whom TAAD may still develop later in life, a systemic score greater than or equal to seven were selected for the SVI's "Phenotype consistent with gene but not highly specific and high genetic heterogeneity" category.

Multiple segregations of a variant with phenotype in affected family members (PP1)

Jarvik & Browning previously published Bayesian-derived guidelines for consideration of a variant's co-segregation with disease in a family [42]. The VCEP initially incorporated this guidance but ultimately determined that the less stringent framework utilized by the Hearing Loss VCEP for autosomal dominant hearing loss [23] was more appropriate for Marfan syndrome. As such, PP1_Supporting is met by the presence of two to three segregations of a given variant with clinical features of Marfan syndrome, PP1_moderate is met with four segregations, and five or more segregations fulfills the PP1_Strong criterion.

Functional evidence supportive of a damaging effect or no effect (PS3, BS3)

Evaluation of functional data should follow the ClinGen SVI's framework for application of PS3 and BS3 [43]. Consistent with this recommendation, the VCEP specifies the types of experimental assays that are valid for assessment of *FBN1* variants as shown in Fig. 3. The assays deemed appropriate include complementary DNA (cDNA) analyses performed in the presence of a nonsense-mediated decay (NMD) inhibitor showing an altered *FBN1* RNA sequence, and in vitro engineered systems showing altered *FBN1* protein or RNA expression, proteolysis, folding, assembly, trafficking, secretion, calcium (Ca^{2+})-binding, matrix deposition, and microfibril fragmentation or catabolism. Functional studies deemed inappropriate for application of PS3 or BS3 include assays that identify non-specifically altered TGF-β signalling or histological hallmarks of medial degeneration, as these are also seen with variation in several other genes associated with hereditary TAAD and are not specific to *FBN1* and Marfan syndrome.

Computational evidence supporting a deleterious effect or no effect on the gene or gene product (PP3, BP4)

The repeated demonstration of the meta-predictor REVEL's [44] high performance, its positive and negative predictive value for *FBN1* missense variants compared to others, and its availability and ease of use resulted in its recommendation as the computational pathogenicity prediction algorithm for evaluation of *FBN1* variants. Based on analyses of known pathogenic missense variants and their respective REVEL scores, PP3 was determined to be applicable for variants with REVEL scores greater than or equal to 0.75. Following the comprehensive analysis by Tian et al. [45], BP4 was determined to be applicable for missense variants with REVEL scores less than or equal to 0.326. For variants with potential impacts to splicing, either PP3 or BP4 should be applied when the computational splicing prediction algorithms GeneSplicer [46], MaxEntScan [47], and NNSplice [48] are concordant on their predictions of either an impact or lack of predicted impact on splicing, respectively.

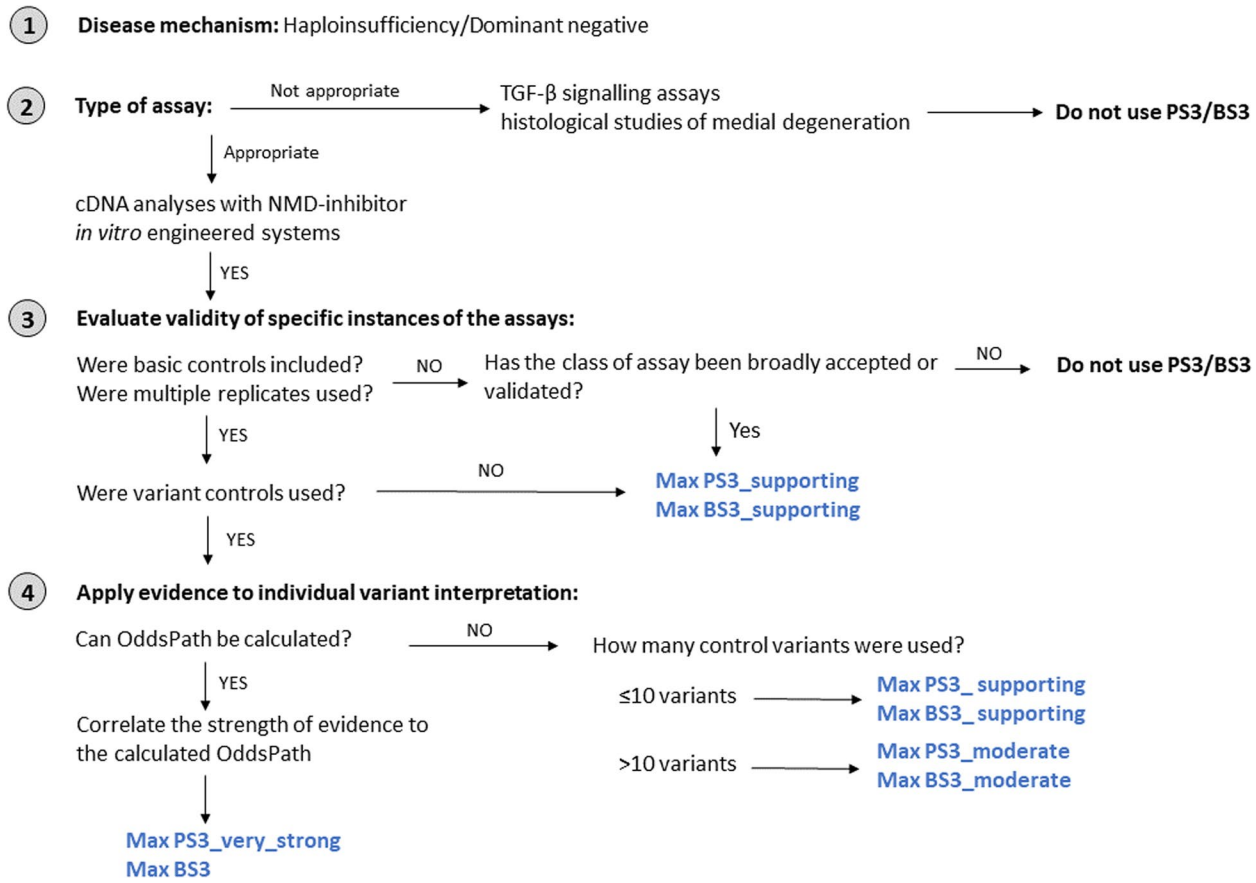

Fig. 3 Flowchart for the adapted PS3/BS3 criteria for functional evidence of damaging effect: the *FBN1* VCEP made minor modifications to the original decision tree developed by ClinGen [43]. The most relevant specification is step 2 for which the VCEP defines the assays deemed appropriate for consideration

Patient's phenotype or family history is highly specific for a disease with a single genetic aetiology (PP4)

Application of the PP4 criterion accounts for an individual under investigation who manifests a phenotype and/or has a family history highly specific to a single gene. The *FBN1* VCEP opted to utilize the revised Ghent criteria for diagnosis of Marfan syndrome [9], stating that PP4 should be applied for variants identified in individuals who meet these well-established and highly specific clinical diagnostic criteria. The VCEP also advises that a laboratory should be cautious but may use their discretion regarding the requirement of a clinical diagnosis in instances in which a variant is identified in a young patient with a highly suspicious phenotype in whom some of the characteristic features of Marfan syndrome may not have yet manifested (e.g. an infant with ectopia lentis and systemic features but a systemic score less than seven and no TAAD).

Variant co-occurs with a pathogenic variant for a fully penetrant disorder (BP2)

The VCEP states that in order to apply the BP2 criterion, one of two scenarios must be fulfilled. The first is that the variant under investigation must have been found in *trans* with a pathogenic *FBN1* variant in at least two distinct cases without the patients manifesting more severe phenotypes than when the variant is present in isolation. Second, BP2 can be applied if a variant under investigation has been shown in *cis* with a pathogenic variant, with the requirement that the pathogenic variant has been previously identified in isolation in an individual with a phenotype consistent with Marfan syndrome.

Pilot testing of rule specification

The pilot study cohort ($n=60$) comprised a wide variety of variant types and characteristics to ensure that a wide breadth of possible evidence types and associated evidence criteria would be addressed during the pilot (Fig. 4A, B). The cohort included multiple variants with

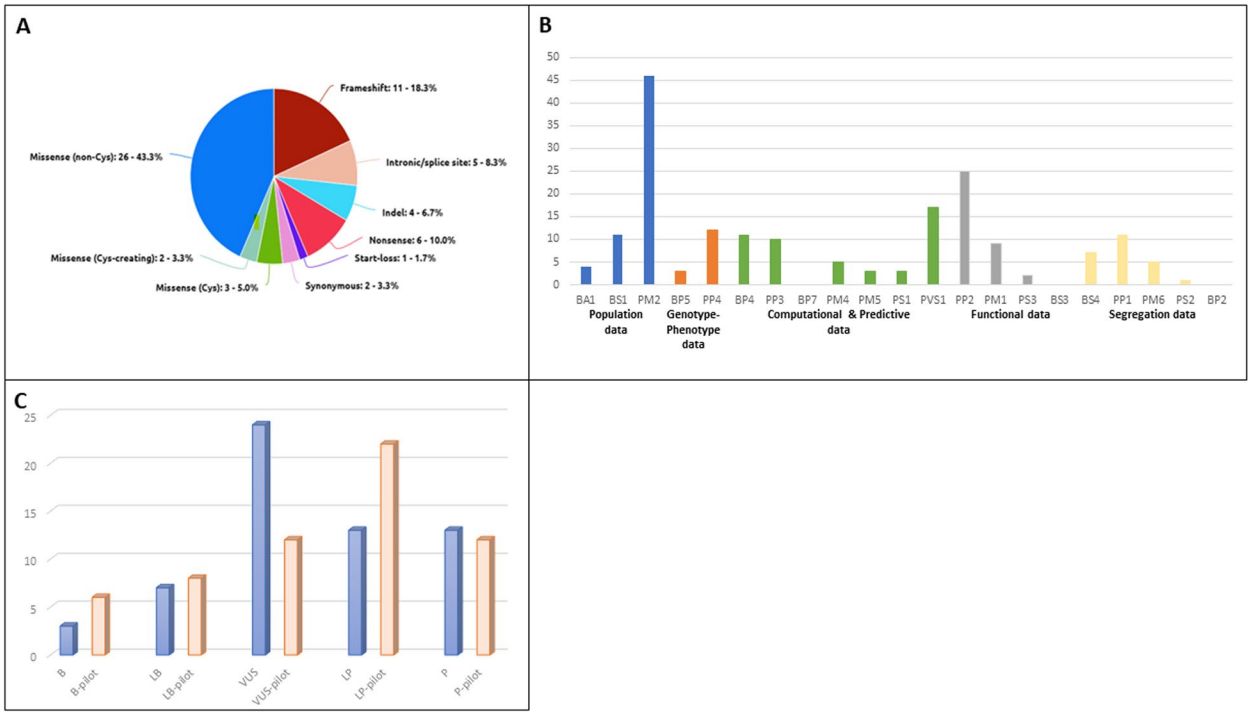

Fig. 4 Results of the *FBN1* VCEP pilot study. **A** Distribution of variant types included in the study. **B** Criteria evaluated during the pilot study. Each colour in the *X*-axis represents a different category of data as defined by the text underneath. The *Y*-axis shows the number of times each criterion was evaluated. **C** Comparison of the classification of the pilot variants according to the referring laboratory (blue) and the classification according to the *FBN1* VCEP (orange)

each of the five classifications (benign, likely benign, VUS, likely pathogenic, and pathogenic) according to the submitting VCEP institutions (Fig. 4C).

Results of the pilot study

In comparing the original institution-made classifications with those achieved using the *FBN1* specifications (Fig. 4C), the number of VUSs reduced from 24 to 12, and the number of benign (3 to 6), likely benign (7 to 8), and likely pathogenic (13 to 22) classifications all increased, with a slight decrease in pathogenic classifications (13 to 12). There were three criteria deemed applicable for *FBN1* that were not used for any variants in the pilot (Fig. 4B): BP7 (synonymous variant with no predicted impact on splicing and occurs at a poorly conserved nucleotide), as there were only two synonymous variants included, and they had predicted aberrant splicing impacts; BS3 (functional study demonstrates no impact of the variant), which will rarely be considered in practice because very few publications exist that experimentally demonstrate an *FBN1* variant's lack of impact; and BP2 (variant co-occurs with a pathogenic variant for a fully penetrant disorder), due to the decision to use the similar BP5 criterion (variant found in a case with an alternate molecular basis for disease) for multiple cases instead.

Concordance between the non-core VCEP institutions and the core team for variant classifications obtained using the *FBN1* specifications was 85.0% (51/60). Of the nine variants with discordant classifications, five represented potentially clinically significant discordance (e.g. VUS vs. likely benign, VUS vs. likely pathogenic) and four represented differences in the degree of confidence (e.g. benign vs. likely benign, pathogenic vs. likely pathogenic). The most prominent causes of classification discordance included differences in usage of population cut-offs due to specifications about the appropriate population in gnomAD [49] to be used for minor allele frequency evaluation, different standards for the application of PP4 (phenotype is highly specific for a single gene) for affected probands, differences in application of PM1 (mutational hotspot or functional domain without benign variation), discordant criteria strength modifications, and discrepancies in classification practices when faced with conflicting variant evidence. These differences were targeted for resolution in the subsequent iteration of the specifications. While each VCEP institution may have encountered slightly different issues with the specifications due to receiving different pilot variants to interpret and classify, in general, most sources of discordance or potential confusion were experienced by

each institution. More detail on the sources of discordance experienced during the pilot study are presented in Supplementary Table 2. Of note, the VCEP classifications reported in Supplementary Table 2 represent the classification determined during the pilot process; these may or may not reflect the ultimate classification obtained when the same variants are formally curated and submitted to ClinVar following ClinGen SVI approval of these specifications, such as in the case newly available data. The final classification as formally curated and published in ClinVar is also shown for the relevant variants in Supplementary Table 2.

Discussion

The process of defining the ACMG-AMP criteria with respect to a single gene or disease is an undertaking of considerable complexity, and any refinements are contingent upon a deep understanding of the gene and its role in pathogenesis. Extensive clinical and bench research has contributed valuable insights into the relationship between *FBN1* variation and Marfan syndrome facilitated the work of the *FBN1* VCEP. The comprehensive understanding of the mutational spectrum, penetrance, and disease prevalence enabled the establishment of appropriate discriminatory minor allele frequency cut-offs. These cut-offs help to effectively identify benign variants and exclude them from further analysis. Additionally, the identification of 22.0% of amino acid positions in the encoded protein as likely functionally important, supported the PM1 evidence code, thereby greatly aiding in the classification of missense variants. These variants may not have an obvious detrimental impact on the gene product compared to variants resulting in haploinsufficiency. Baudhuin et al. emphasized the significance of incorporating gene-specific knowledge into the interpretation of *FBN1* variants in ClinVar [40]. This includes considering minor allele frequency cut-offs and recognizing the importance of conserved and functionally important non-cysteine residues throughout the gene, the latter of which was critical in the interpretation and classification of non-cysteine missense variants. The specifications outlined in this publication address these concerns and aim to minimize the potential for misclassification.

The discordance in classification observed during the pilot study can be attributed to variations in the interpretations and application of certain evidence criteria. These discrepancies include differences in minor allele frequency thresholds, determining functional domains or mutational hotspots, and evaluating relevant phenotypes and their specificity to *FBN1*. Amendola et al. have reported similar findings when assessing the usage of the ACMG-AMP criteria across multiple laboratories [19, 20]. Their research revealed that modifications to criteria strength levels and selective application of certain criteria, specifically those involving subjective judgement or discretion, resulted in discordant classifications. In the case of *FBN1*, the VCEP has successfully worked towards minimizing the potential differences in laboratory-specific utilization of the ACMG-AMP criteria, thereby reducing classification discordance.

The pilot study demonstrated these rule specifications' utility for reducing the quantity of variants given VUS classifications in favour of more benign, likely benign, and likely pathogenic classifications. The resultant increase in the rate of diagnostic genetic testing will have significant medical management and family planning implications for probands and their family members [12, 14–16]. The reduction of VUS in favour of (likely) benign genetic testing results is also desirable, as negative results provide clinicians with justification for pursuing additional genetic testing and eliminate the need to spend both time and financial resources on pursuing segregation analyses for VUSs that may be of relatively low clinical suspicion [50]. Therefore, it is of utmost importance to recontact the clinicians who requested the genetic investigations to pass on the information regarding variant reclassification to their patients. However, the protocols established for this purpose will depend on each institution and fall outside the scope of this work. Further, the return of VUSs from genetic testing has been demonstrated to have variable psychological impacts on some patients [51]; the reduction of VUSs could feasibly have the additional benefit of reducing occurrences of psychological distress for some individuals and families.

The collaborative nature of the VCEP's curation effort highlights the importance of improved sharing of data and processes between laboratories or other institutions involved in variant classification. As each VCEP institution contributes their own internal data for individual variant classification, the nine institutions are effectively sharing data in an effort to reach a consensus classification. This is highly analogous to the work of Harrison et al. who demonstrated that data sharing between clinical laboratories, particularly of clinical data related to probands and their families, was extremely effective in reducing classification discrepancies and resulted in vastly improved classification concordance [52, 53]. VCEPs are uniquely suited for this type of high-impact data sharing; most VCEPs' constituent institutions and experts have prolific histories of managing individuals with the disease(s) of interest and thus possess an abundance of useful clinical data, and there are already-established lines of communication and methods for data sharing that ease the potential burden associated with this process. Successful collaborations to reach consensus classifications also emphasize the importance at the

institutional level of contributing to data sharing initiatives like ClinVar [7], DECIPHER [54], Leiden Open Variation Database [55], and Universal Mutation Database [56] so that laboratorians and clinicians can access as much relevant clinical data as possible and employ these interpretation specifications to their fullest extent.

Limitations

We recognize that several of the criteria, which can be crucially important to a variant's curation, are dependent on the presence of robust clinical information, including for assessment of the applicability and strength of PS4, PS2/PM6, and PP1, and the appropriateness of PP4. Diagnostic laboratories do not always have detailed clinical data for a patient or family when assessing a variant, and these specifications cannot provide clarity beyond the available data. Further, due to the variable expressivity and often age-dependent penetrance of Marfan syndrome features, there will always be an inherent limitation in the interpretation of some *FBN1* variants based on the patients' clinical presentations, as well as a potential bias in evaluating variant re-interpretation surrounding the ages between initial and re-interpretation. Additionally, these criteria have been developed specifically to curate variants in *FBN1* causing Marfan syndrome and may not be applicable to other diseases caused by variation in *FBN1*.

Conclusions

The *FBN1* VCEP introduced 14 modifications to the original 28 ACMG-AMP variant classification criteria. Establishing these specific adaptations for *FBN1* provides a framework to improve classification concordance among clinical laboratories which will ultimately result in an improvement of clinical care for patients with Marfan syndrome.

The VCEP will maintain a monthly meeting schedule to review variants that have been pre-curated by one of two biocurators in collaboration with a rotating VCEP institution. The final classification of these variants will be determined through group consensus among all members. The primary focus of curation efforts will be on variants with conflicting interpretations in ClinVar. Currently, the VCEP has completed curation and ClinVar submission for 120 *FBN1* variants, with an estimated annual curation rate of 120 variants. We acknowledge that upcoming updates to the technical standards for sequence variant interpretation will require adjustments to these rule specifications to align with standard practices. This challenge is faced by all VCEPs, and guidance from the ClinGen SVI will likely be relied upon. We are confident that the current rule specifications can be easily transferred to the new framework, as the fundamental principles for *FBN1* variant assessment will remain unchanged. Furthermore, this forthcoming publication will provide an opportunity for reassessment of the *FBN1* specifications as outlined here. The *FBN1*-specific guidelines for variant classification and curation have already demonstrated success in the pilot phase and in practice. They have introduced a standardized interpretation framework and improved classification agreement among clinical laboratories, ultimately leading to enhanced clinical care for this patient population. However, variant curation is an evolving effort in continuous need for reassessment and this process will persist over time.

Abbreviations

ACMG/AMP	American College of Medical Genetics and Genomics and the Association for Molecular Pathology
ClinGen	Clinical Genome Resource
EGF	Epidermal growth factor
SVI	ClinGen Sequence Variant Interpretation Working Group
TAAD	Thoracic aortic aneurysm and dissection
VCEP	Variant curation expert panel
VUS	Variant of unknown significance

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions

MR, JDB, and LMM organized the panel meetings in stages 1–3. They built and analysed the online surveys. JDB and LMM organized and coordinated the pilot study. All authors participated in the monthly discussions and contributed variants to the pilot study. LB, KK, MLK, PA, and NH reviewed all surveys and the results of the pilot study as part of the core team. AD and LMM drafted the manuscript and the rebuttal to the reviewers. All authors read and approved the final manuscript.

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Data availability

The datasets supporting the conclusions of this article are included within the article and supplementary files. All curated variants have been deposited in the ClinVar database[7] with 3-star "Expert panel" review status (https://www.ncbi.nlm.nih.gov/clinvar/). The results of the conducted surveys and the specific report on the pilot study process and progress can be made available upon request. Some data included in the original files could compromise individual privacy.

Declarations

Ethics approval and consent to participate

This research was performed according to the principles of the Helsinki Declaration. It only includes de-identified data, and therefore ethical approval is not applicable.

Competing interests

The authors declare that they have no competing interests.

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