Received: 27 June 2022 | Revised: 2 September 2022 | Accepted: 6 October 2022

DOI: 10.1002/humu.24482

RESEARCH ARTICLE

Human Mutation HGVS HUMAN GENOME VARIATION SOCIETY WILEY

Specifications of the ACMG/AMP variant curation guidelines for myocilin: Recommendations from the clingen glaucoma expert panel

Kathryn P. Burdon[1] | Patricia Graham[1,2] | Johanna Hadler[3,4] | John D. Hulleman[5] | Francesca Pasutto[6] | Erin A. Boese[7] | Jamie E. Craig[3] | John H. Fingert[7] | Alex W. Hewitt[1] | Owen M. Siggs[3,8] | Kristina Whisenhunt[9] | Terri L. Young[9] | David A. Mackey[1,2] | Andrew Dubowsky[4] | Emmanuelle Souzeau[3]

[1]Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia

[2]Centre for Ophthalmology and Vision Sciences, Lions Eye Institute, University of Western Australia, Perth, Western Australia, Australia

[3]Department of Ophthalmology, Flinders University, Flinders Medical Centre, Adelaide, South Australia, Australia

[4]SA Pathology, Adelaide, South Australia, Australia

[5]Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, Texas, USA

[6]Institute of Human Genetics, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany

[7]Department of Ophthalmology and Visual Sciences, University of Iowa, Iowa City, Iowa, USA

[8]Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia

[9]Department of Ophthalmology and Visual Sciences, University of Wisconsin-Madison, Madison, Wisconsin, USA

Correspondence
Emmanuelle Souzeau, Department of Ophthalmology, Flinders University, 1 Flinders Drive, Bedford Park, SA, 5042, Australia.
Email: Emmanuelle.souzeau@flinders.edu.au

Funding information
Hospital Research Foundation; Research to Prevent Blindness; National Institutes of Health; National Health and Medical Research Council

Abstract

The standardization of variant curation criteria is essential for accurate interpretation of genetic results and clinical care of patients. The variant curation guidelines developed by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) in 2015 are widely used but are not gene specific. To address this issue, the Clinical Genome Resource (ClinGen) Variant Curation Expert Panels (VCEP) have been tasked with developing gene-specific variant curation guidelines. The Glaucoma VCEP was created to develop rule specifications for genes associated with primary glaucoma, including *myocilin* (*MYOC*), the most common cause of Mendelian glaucoma. Of the 28 ACMG/AMP criteria, the Glaucoma VCEP adapted 15 rules to *MYOC* and determined 13 rules not applicable. Key specifications included determining minor allele frequency thresholds, developing an approach to counting probands and segregations, and reviewing functional assays. The rules were piloted on 81 variants and led to a change in classification in 40% of those that were classified in ClinVar, with functional evidence influencing the classification of 18 variants. The standardized variant curation guidelines for *MYOC* provide a framework for the consistent application of

the rules between laboratories, to improve *MYOC* genetic testing in the management of glaucoma.

KEYWORDS
genetic testing, juvenile open-angle glaucoma, MYOC, primary open-angle glaucoma, variant classification, variant curation expert panel, variant interpretation

1 | INTRODUCTION

Accurately interpreting sequence variants is a key component in genetic diagnosis. In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published guidelines to standardize the interpretation and classification of sequence variants in genes associated with Mendelian diseases (Richards et al., 2015). The guidelines have since been widely adopted by the genetics community. The ACMG/AMP guidelines were intended to be broadly applicable and were designed to provide flexibility and adaptability. However, recent studies have reported discrepancies and inconsistency in variant interpretation and classification between laboratories using the guidelines (Amendola et al., 2016; Amendola et al., 2020; Harrison et al., 2017). This highlights the need to develop further expert-led guidance specific to the gene or disease.

The Clinical Genome Resource (ClinGen, www.clinicalgenome.org) aims to define the clinical relevance of genes and variants through collaborative international efforts to improve genetic diagnosis. Within ClinGen, the Variant Curation Expert Panels (VCEP) are tasked with developing gene-specific variant curation guidelines (Rivera-Muñoz et al., 2018). The Glaucoma VCEP was formed to review the evidence and curate variants in genes associated with primary glaucoma.

Glaucoma refers to a heterogeneous group of disorders characterized by progressive optic neuropathy with associated visual field defects (Casson et al., 2012). Primary open-angle glaucoma (POAG; MIM# 137750) is the most common type of glaucoma and is characterized by an open anterior chamber angle and no developmental defects or other underlying disease (American Academy of Ophthalmology Preferred Practice Pattern Glaucoma Committee, 2020). POAG with an early age of onset is called juvenile open-angle glaucoma (JOAG) and is usually defined as age at diagnosis <30–40 years (but after age 5) (Turalba & Chen, 2008).

Pathogenic variants in the *myocilin* gene (*MYOC*, formerly *trabecular meshwork-induced glucocorticoid response gene* or *TIGR*; MIM# 601652) are the most common cause of Mendelian JOAG and POAG (Stone et al., 1997). They account for 8%–36% of JOAG (Shimizu et al., 2000; Vincent et al., 2002; Wiggs et al., 1998) and 2%–4% of POAG (Fingert et al., 1999; Souzeau et al., 2013). In high-risk individuals with a strong family history of glaucoma, cascade genetic screening can allow targeted clinical screening and facilitate early diagnosis and treatment of glaucoma (Souzeau et al., 2017). The *MYOC* database compiles all published variants and currently contains 358 variants (www.myocilin.com, accessed 7 June 2022) (Hewitt et al., 2008). Glaucoma-causing *MYOC* variants are transmitted in an autosomal dominant manner, often with age-related, incomplete penetrance. The most common pathogenic *MYOC* variant is p.Gln368Ter, accounting for 1.6%–2.6% of individuals with POAG (Fingert et al., 1999; Souzeau et al., 2013). This variant is present in as many as 1/300 individuals in reference databases (highest prevalence reported in https://gnomad.broadinstitute.org, version 2.1.1, accessed 7 June 2022), and previous studies support a common European founder effect (Baird et al., 2003; Fingert et al., 1999).

MYOC contains 3 exons and encodes a 504 amino acid protein. Most reported disease-causing variants are located in the olfactomedin domain coded by exon 3 (amino acid residues 246–502) (Hewitt et al., 2008). *MYOC* whole gene deletions are not associated with disease in humans (Wiggs & Vollrath, 2001) and the absence of disease with heterozygous or homozygous premature termination variants in the first two exons that would be expected to be subject to nonsense-mediated decay in humans (Lam et al., 2000) or mouse models (Kim et al., 2001) strongly argues against haploinsufficiency as a disease mechanism. The mechanism by which *MYOC* variants cause POAG is not fully understood but current evidence supports a toxic gain-of-function mechanism. Evidence suggests that *MYOC* variants lead to misfolded protein (Burns et al., 2011; Liu & Vollrath, 2004; Vollrath & Liu, 2006) and that the mutant protein is not secreted extracellularly (Caballero et al., 2000; Gobeil et al., 2004; Izumi et al., 2003; Jacobson et al., 2001; Nakahara & Hulleman, 2022; Vollrath & Liu, 2006; Zadoo et al., 2016). Moreover, mutant MYOC protein is not folded correctly in the endoplasmic reticulum, leading to accumulation of insoluble aggregates inside the trabecular meshwork cells, (Caballero & Borrás, 2001; Liu & Vollrath, 2004) inducing the unfolded protein response and resulting in endoplasmic reticulum stress-induced apoptosis (Yam et al., 2007).

The Glaucoma VCEP was created with the goal of generating specifications to the ACMG/AMP guidelines for *MYOC*. Herein, we describe the process of developing specified variant classification rules for *MYOC*, validating the rules on a set of variants, and curating *MYOC* variants with submission to ClinVar with expert panel review status. ClinVar is a freely accessible, public archive which reports the relationships among human variations and phenotypes, with supporting evidence (Landrum et al., 2018).

2 | MATERIALS AND METHODS

2.1 | VCEP membership and framework

The Glaucoma VCEP sits within the ClinGen Ocular Clinical Domain Working Group. Members were selected to provide broad expertise including clinical ophthalmology and research, with an emphasis on glaucoma as a subspecialty, molecular biology, molecular diagnostics, and genetic counseling. Representation from multiple countries was also sought, with panel members from Australia, the United States and Germany. Details of composition and membership can be found on the VCEP website (https://clinicalgenome.org/affiliation/50053/).

2.2 | Project design

The Glaucoma VCEP met regularly through video and telephone conferences to review the ACMG/AMP guidelines (Richards et al., 2015) and discuss changes and specifications relevant to *MYOC* POAG/JOAG. The abbreviations for each rule and its original specification can be found in Table 1 (Table S1 for rules deemed not applicable). Individuals or small working groups were convened to gather relevant evidence and information for each rule, and this was presented to the VCEP for discussion. Consensus decisions were reached on the conference call and minutes circulated with the accompanying information to members unable to attend.

Draft rules were applied to a list of 81 pilot variants, which included the majority of variants with previous classifications from ClinVar, to assess how the refined criteria performed against previous classifications. The list covered variants from all three exons, different categories of variants (truncating variants [nonsense, frameshift], missense, synonymous, in-frame indels), different variant classifications (benign [B]/likely benign [LB], pathogenic [P]/likely pathogenic [LP], and variants of uncertain significance [VUS]) and all variants with conflicting evidence from ClinVar). Additional variants were included to ensure that all criteria were applied in the pilot. All variants with functional evidence were included to review the level of strength of these studies. Two biocurators applied the draft rules to each pilot variant (P.G. and J.H.), noting issues that arose while doing so. These were discussed by a core pilot working group consisting of a genetic counselor (E.S.), an ophthalmologist (D.A.M.), a molecular geneticist (K.P.B.), a clinical scientist (A.D.), and the biocurators. This group made recommendations for further modifications and refinements to the rules for discussion by the full VCEP in an iterative process. The final set of rules was disseminated to all VCEP members for comment before submission to the ClinGen Sequence Variant Interpretation (SVI) Working Group, which provides harmonization across Clinical Domain Working Groups.

Variant curations were performed by the biocurators in the ClinGen Variant Curation Interface (https://curation.clinicalgenome.org/) and were reviewed by a core approval member (E.S.) for initial assessment, with additional review by two more core approval members (A.D., K.P.B., F.P., K.W.) for final approval. A summary of approved variant interpretations was then sent to all VCEP members for feedback. All variant classifications, criteria applied and supporting evidence were submitted to ClinVar with expert status (3-star level). Variants were annotated using GenBank reference sequence NM_000261.2 and NP_000252.1 using genome build GRCh37/hg19.

2.3 | Data sources

Publicly available variant data were obtained between June and November 2021 from ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/int) and the Myocilin database (www.myocilin.com) (Hewitt et al., 2008), which compiles all variants reported in the literature. Additional published and unpublished case-level data were used for variant curation from research databases of VCEP panel members including J.E.C. (The Australian and New Zealand Registry of Advanced Glaucoma [ANZRAG]) (Souzeau et al., 2013), D.A.M. (The Glaucoma Inheritance Study of Tasmania [GIST]) (Mackey et al., 2019), F.P., and T.Y. All variants are publicly listed in the Myocilin database. We defined JOAG as a diagnosis at ≤40 years old.

2.4 | Rule specific approaches

2.4.1 | Population data (BA1, BS1, and PM2)

Inheritance pattern, disease prevalence, allelic contribution, and penetrance information were gathered from the literature and used in the Whiffin/Ware calculator (https://cardiodb.org/allelefrequencyapp/) (Whiffin et al., 2017) to establish minor allele frequency thresholds for BA1, BS1 and PM2 specific to *MYOC*.

2.4.2 | Proband counts (PS4)

To set thresholds for the number of probands to indicate a significant increase in prevalence of a variant in affected individuals compared to controls, odds ratios (ORs) were calculated for hypothetical variants with varied numbers of observed probands and minor allele counts in population databases, similar to the approach taken by Kelly et al. (2018). Sample size was set to 3,522 cases, (based on the number of POAG/JOAG probands with *MYOC* results in the ANZRAG/GIST databases in March 2021) and 125,748 unrelated reference samples (as recorded in gnomAD v2.1.1 at the same date). Given that ANZRAG predominantly contains individuals of European descent, the process was repeated with 3,277 cases and 56,885 unrelated reference samples, representing the Non-Finnish European (NFE) cohort in gnomAD. Proband count for Supporting, Moderate and Strong thresholds were selected as those that gave rise to ORs of 10, 30, and 100, as per Kelly et al. (2018).

TABLE 1 MYOC rule specifications

Pathogenic criteria	ACMG criteria	Strong	Moderate	Supporting	Specification
Population data					
PM2	Absent in population databases.			Allele frequency ≤0.0001 in population databases.	Highest allele frequency in population databases should be used. Only applies to populations with ≥ 10,000 alleles.
PS4	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.	≥ 15 probands from multiple independent studies	≥ 6 probands from multiple independent studies	≥ 2 probands from multiple independent studies	Probands counted should be clinically assessed and have a diagnosis of JOAG or POAG. PM2_Supporting must be met.
Computational and predictive data					
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product.			Applies to missense variants with a REVEL score of ≥ 0.7	
PM5	Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.		Same residue as a previously established P variant (assessed independently of PM5) or 2 previously established LP variants (both assessed independently of PM5)	Same residue as a previously established LP variant (assessed independently of PM5)	The novel change must not affect splicing (SpliceAI ≤ 0.2), must meet PP3 and have a Grantham score equal or greater than the previously established P/LP variants.
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	Same amino acid change as a previously established P variant (assessed independently of PS1)	Same amino acid change as a previously established LP variant (assessed independently of PS1)		The novel change must not affect splicing (SpliceAI ≤ 0.2).
PM4	Protein length changes as a result of in-frame deletions/insertions in a nonrepeat region or stop-loss variants.		In-frame del/ins and truncating variants involving > 10% of the protein	In-frame del/ins and truncating variants involving ≤ 10% of the protein	Variant must be located within the conserved olfactomedin domain (AA 246-502).
Functional data					
PS3	Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product.	Assays with OddsPath > 18.7	Assays with OddsPath > 4.3	Assays with OddsPath > 2.1	Applies to the following functional studies: Solubility or secretion assays or animal models that replicate the glaucoma phenotype.

(Continues)

TABLE 1 (Continued)

Pathogenic criteria	ACMG criteria	Strong	Moderate	Supporting	Specification
Segregation data					
PP1	Cosegregation with disease in multiple affected family members in a gene definitively known to cause the disease.	≥7 meioses in >1 family	≥ 5 meioses	≥ 3 meioses	BA1 and BS1 must not be met. Only genotype-positive/phenotype-positive and obligate carriers/phenotype-positive individuals should be counted as segregations. Phenotype-positive need to be clinically assessed and either have a diagnosis of glaucoma or suspicious signs of glaucoma.
De novo data					
PS2/PM6	De novo in a patient with disease and no family history.	≥ 2 confirmed de novo in JOAG	≥ 2 confirmed de novo in POAG or 1 confirmed de novo in JOAG or ≥2 assumed de novo in JOAG	1 confirmed de novo in POAG or ≥ 2 assumed de novo in POAG or 1 assumed de novo in JOAG	Use proposed SVI point recommendations for "phenotype consistent with the gene but not highly specific and with high genetic heterogeneity" for POAG and "phenotype consistent with gene but not highly specific" for JOAG. Both maternity and paternity need to be proven for confirmed de novo variants. Parents need to be clinically assessed and not have a diagnosis of glaucoma.

Benign criteria	ACMG criteria	Stand alone	Strong	Moderate	Supporting	Specification
Population data						
BA1/ BS1	Allele frequency is greater than expected for disorder.	**BA1**Allele frequency ≥ 0.01 in population databases	**BS1**Allele frequency ≥ 0.001 in population databases. Does not apply to p.Gln368Ter.			The highest allele frequency in population databases should be used. Variant must be present in ≥ 5 alleles in any validated general continental population data set of at least 2,000 observed alleles.
Computational and predictive data						
BP4	Multiple lines of computational evidence suggest no impact on gene/gene product.				Applies to missense variants with a REVEL score ≤ 0.15 or synonymous or noncoding variants with CADD ≤ 10 AND SpliceAI ≤ 0.2	

TABLE 1 (Continued)

Benign criteria	ACMG criteria	Stand alone	Strong	Moderate	Supporting	Specification
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.				Applies to synonymous variants or noncoding variants with no impact on splicing (SpliceAI ≤ 0.2) AND GERP < 0 for conservation	
Functional data						
BS3	Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing.			Assays with OddsPath < 0.23	Assays with OddsPath < 0.48	Applies to variants showing solubility and secretion in functional assays.

Abbreviations: AA, amino acid; B, benign; JOAG, juvenile open-angle glaucoma; LB, likely benign; LP, likely pathogenic; P, pathogenic; POAG, primary open-angle glaucoma; SVI, ClinGen Sequence Variant Interpretation Working Group.

2.4.3 | Segregation data (PP1)

Thresholds for the number of meioses required to apply PP1 at each level of strength were taken from Kelly et al. (2018) and Jarvik and Browning (2016) and the classification outcomes were compared. The thresholds giving the most conservative classification were selected.

2.4.4 | Computational predictive tools (PP3, BP4, and BP7)

Rare Exome Variant Ensemble Learner (REVEL) (Ioannidis et al., 2016), Functional Analysis Through Hidden Markov Models (FATHMM) (Shihab et al., 2013) and Combined Annotation-Dependent Depletion (CADD) (Kircher et al., 2014; Rentzsch et al., 2019) scores were calculated for all non-synonymous and synonymous (where possible) *MYOC* variants reported in ClinVar (July 2021) and compared to each other and against the classifications reported in ClinVar. HSF (Desmet et al., 2009), MaxEntScan (Yeo & Burge, 2004), NeuralNetwork (Reese et al., 1997), and SpliceAI (Jaganathan et al., 2019), were used to predict effects on splicing for synonymous variants. Tools and threshold selection were made based on the ability of each tool to discriminate variants previously classified in ClinVar as P/LP or B/LB, combined with the recommendations for interpreting output from each tool.

3 | RESULTS

3.1 | Summary of rule specifications

The final rule specifications adapted from the ACMG/AMP guidelines for *MYOC* by the Glaucoma VCEP were approved by the SVI Working Group on 12 October 2021. These are summarized in Table 1 and are available online (https://www.clinicalgenome.org/affiliation/50053/). Of the 28 criteria, 11 were determined not applicable to *MYOC* (PVS1, PM1, PM3, PP2, PP4, BS2, BS4, BP1, BP2, BP3, BP5, Supp Tables S1) and 2 were excluded based on previous SVI recommendations (PP5, BP6) (Biesecker & Harrison, 2018). The remaining 15 rules were all modified to be specific to *MYOC* and/or the associated phenotype. The level of strength was modified for two rules (PM2, BS3) and additional levels added for 8 (PS1, PS2/PM6, PS3, PS4, PM4, PM5, PP1, BS3).

3.2 | Population data

3.2.1 | Allele frequency data (BA1, BS1, and PM2_Supporting)

BA1, BS1, and PM2 apply to the frequency of a variant in a control or general population. Allele frequency thresholds for BA1 (allele

frequency >5%) and BS1 (allele frequency greater than expected for the disorder) specific to *MYOC* were calculated with the Whiffin/Ware calculator.

The highest prevalence for POAG is found in African populations (1/24) (Quigley & Broman, 2006; Tham et al., 2014). The maximum proportion of individuals with JOAG or POAG potentially attributable to a single allele corresponds to the *MYOC* p.Gln368Ter variant, (the most frequently reported variant) with a prevalence of 1.6%–2.6% depending on the study (Fingert et al., 1999; Siggs et al., 2021; Souzeau et al., 2013). Analysis of the penetrance of p.Gln368Ter showed that it was much lower in a population-based study (UK Biobank [UKB], 7.6%) than in family-based studies (ANZRAG/GIST, 56%) (Han et al., 2019), although both studies have their own biases. The UKB relied in part on self-report for diagnosis in addition to elevated intraocular pressure or ICD-9/ICD-10 coding. It is well established that at least 50% of individuals with glaucoma are undiagnosed (Mitchell et al., 1996; Soh et al., 2021), so reliance on self-report can lead to underestimation of penetrance. More recent analyses indicate the penetrance of the p.Gln368Ter variant in UKB is around 25% (Zebardast et al., 2021). Conversely, the penetrance in family-based studies may be inflated if individuals with glaucoma are more likely to participate than unaffected family members. Using the prevalence of POAG in Africans, a maximum allelic contribution at 2.6%, and a conservative estimate for the penetrance at 7.6%, we calculated a maximum credible population allele frequency for BA1 at 0.007, which was rounded up to 0.01 (1%). Using the same prevalence and maximum allelic contribution, and a more realistic estimate of the penetrance at 56%, we calculated an allele frequency threshold for BS1 at 0.001 (0.1%). We adopted the SVI recommendations that the variant be present in ≥5 alleles in any validated general continental population data set of at least 2000 observed alleles (Ghosh et al., 2018; Sequence Variant Interpretation Working Group, 2020), to prevent using less well-characterized populations in the assessment of BA1 and BS1.

An exception was applied to the BS1 rule for variant p.Gln368Ter, based on its penetrance and the presence of a common disease haplotype in all carriers (Baird et al., 2003). Its allele frequency was 0.0025 in the UKB (Han et al., 2019) and 0.001588 in Non-Finnish Europeans in gnomAD, with the highest allele frequency at 0.003344 in Finnish Europeans, which were all above the BS1 threshold established. However, *MYOC* p.Gln368Ter is a definitively established pathogenic variant (Fingert et al., 1999; Jacobson et al., 2001; Zhou & Vollrath, 1999), displaying variable expressivity (Craig et al., 2001) and reduced penetrance (Han et al., 2019).

PM2 was initially defined by the ACMG/AMP guidelines as the absence of the variant from population databases. Although most are absent, *MYOC* pathogenic variants may be present as POAG is a common complex disease with late onset and age-related penetrance, and glaucoma phenotypes are not actively excluded from gnomAD. We therefore decided to allow the presence of *MYOC* variants in population databases for PM2. The filtering allele frequency for PM2 was set one order of magnitude lower than BS1 at 0.0001 (0.01%). PM2 only applies to populations of ≥ 10,000 alleles, to prevent the occurrence of a variant by chance in a small population subset. The Glaucoma VCEP endorsed the SVI recommendation to decrease the weight of PM2 to a Supporting level (Sequence Variant Interpretation Working Group, 2020) and recommended using the highest allele frequency in population databases when assessing BA1, BS1, and PM2_Supporting.

3.2.2 | Phenotype data (PS4, PS4_Moderate, and PS4_Supporting)

PS4 relates to the prevalence of the variant in affected individuals (cases) compared to unaffected controls. Reliable case-control data in individual studies was limited for most *MYOC* variants, with the size of the control cohort often not large enough for a reliable estimate of frequency. Therefore, we adopted the "proband counting" approach recommended by the ACMG/AMP guidelines, which allows counting of probands across multiple independent studies for a "quasi case-control study."

All probands counted must be clinically assessed and have had a diagnosis of JOAG or POAG. To apply PS4, BA1 and BS1 must not be met to avoid counting the occurrence of common variants in affected individuals. PM2_Supporting must be met to indicate a rare allele. Efforts should be made to ensure that probands counted are from independent cohorts and probands should not be counted toward PS4 if uncertainty remains (e.g., published by same groups or authors).

We calculated proband count thresholds at which statistical significance would be reached when compared to a large reference data set such as gnomAD. Evaluation of the ANZRAG/GIST cohorts as a basis for calculations revealed that no pathogenic variants from the cohorts were present at more than 6 alleles in gnomAD (excluding p.Gln368Ter). Using the presence of 6 alleles in gnomAD, ORs of 10, 30, and 100, as per Kelly et al. (2018) were reached for 2, 6, and 17 probands (Table S2). Calculations on cases and controls from a European-only population gave similar thresholds of 2, 6, and 18 probands, when 3 alleles were present in the reduced gnomAD data set (Table S2). Based on similar results to Kelly et al., the same thresholds of 2, 6, and 15 probands for Supporting, Moderate and Strong were selected for the application of PS4.

3.3 | Computational and predictive data

3.3.1 | Computational predictive tools (PP3, BP4, and BP7)

PP3 and BP4 require multiple lines of computation evidence predicting pathogenic or benign characteristics respectively while BP7 relates specifically to non-conserved synonymous variants with no evidence of an effect on splicing. Evaluation of a range of computational predictive tools revealed overlap in the evidence used to make predictions. In order not to overstate the importance of

computational evidence, and in line with reports that a lower rate of concordance is achieved when multiple software tools are used (Ghosh et al., 2017), we decided to use a single tool that collates multiple sources of computational evidence, and where necessary, supplement that with additional sources of evidence.

A comparison of REVEL, FATHMM, and CADD was undertaken for nonsynonymous variants deposited in ClinVar. FATHMM was optimized towards assessing noncoding variants and was discounted. Comparison of the REVEL (https://sites.google.com/site/revelgenomics/downloads) and CADD (Version 1.6; https://cadd.gs.washington.edu/snv) scores revealed that REVEL was more conservative (Figure S1). Multiple benign variants and VUS had CADD scores >20, but REVEL scores <0.5; however, there was broad agreement between the tools at the pathogenic end. Based on this comparison, and the sensitivity/specificity reported for REVEL when predicting pathogenicity of ClinVar variants (Ioannidis et al., 2016), the Glaucoma VCEP recommended defining PP3 as REVEL ≥0.7 (58% sensitivity and 96% specificity) and BP4 as REVEL ≤0.15 (55% sensitivity and 95% specificity) for missense variants, similar to other VCEPs curating variants for autosomal dominant diseases (Luo et al., 2019; Oza et al., 2018).

Several criteria require specific evaluation of potential splicing effects. REVEL considers splicing effects but is unable to score synonymous variants. We evaluated several splice prediction tools for this purpose, seeking one that was freely available and straightforward to use. SpliceAI (https://spliceailookup.broadinstitute.org/) (Jaganathan et al., 2019) met these criteria and had clear interpretation guides in the reporting literature. We did not find any evidence in the literature of *MYOC* variants believed to act through altered splicing and all 19 synonymous variants reported in ClinVar at the time of assessment were classified as B/LB, VUS or conflicting interpretations. All of these had SpliceAI scores <0.2 for all splicing measures, consistent with no *in silico* evidence of effects on splicing. Therefore, we chose SpliceAI scores ≤ 0.2 for assessing splicing effects of synonymous variants.

BP4 requires multiple lines of computational evidence suggesting no impact on gene product. As CADD scores can be calculated for any type of variant and incorporate multiple lines of evidence, we specified that a CADD score of ≤10 and SpliceAI score ≤0.2 were required to apply BP4 to synonymous variants. For the application of BP7 for synonymous variants, we specified a GERP score <0 (https://genome.ucsc.edu/cgi-bin/hgGateway), indicating the nucleotide is not highly conserved, as well as SpliceAI ≤0.2. BP4 and BP7 extend to noncoding variants; however, based on the disease mechanism and the absence of evidence supporting pathogenicity of intronic/noncoding variants, curation of these variants will be deprioritized.

3.3.2 | Protein length changing variants (PM4 and PM4_Supporting)

The Glaucoma VCEP decided that PM4 (protein length changes due to in-frame deletions/insertions and stop losses) would be more appropriate for truncating variants in the last exon that are predicted to escape nonsense-mediated decay, instead of PVS1, which is intended for loss-of-function (LoF) variants. We decided to expand PM4 to any protein length-changing variants, including truncating variants, found within the conserved olfactomedin domain, which is located in exon 3 (aa 246-502). Variants occurring after the last 50bp of exon 2, equating to aa227 would be expected to escape nonsense-mediated decay. The ACMG/AMP guidelines emphasized that larger deletions, insertions or extensions would be considered stronger evidence toward pathogenicity. Therefore, we decided to apply PM4 at a Moderate level if the protein length-changing variant was involving ≥10% of the protein and at a Supporting level if involving <10% of the protein.

3.3.3 | Variants affecting the same amino acid residue (PS1, PS1_Moderate, PM5, and PM5_Supporting)

PS1 applies to a novel nucleotide change leading to the same amino acid residue while PM5 applies to a different missense change. There was only one variant listed in the *MYOC* database that consists of a different nucleotide leading to the same amino acid (p.Asn480Lys caused by c.1440C>A and c.1440C>G). There were 24 variants in the *MYOC* database with 2 different missense variants at the same residue and 6 with >2 different missense variants at the same residue. The VCEP decided to apply different levels of strength to PS1 and PM5 depending on the pathogenicity of the previously established variant. PS1 applies to the same amino acid change as a previously established P variant while PS1_Moderate applies to the same amino acid change as a previously established LP variant. Similarly, PM5 applies to the same residue as a previously established P variant or to 2 previously established LP variants while PM5_Supporting applies to the same residue as a previously established LP variant. The previously established P or LP variants need to reach their classification without the use of PS1 or PM5. In line with the ACMG/AMP guidelines, the novel change must not affect splicing for PS1 or PM5 to apply (as assessed by SpliceAI with a score ≤ 0.2). Additionally, to apply PM5, the variant must meet PP3 and have a Grantham score equal or greater than the previously established P or LP variant to ensure the novel amino acid is predicted to impact function.

3.4 | Functional data (PS3, PS3_Moderate, PS3_Supporting, BS3_Moderate, and BS3_Supporting)

PS3 applies to functional evidence from well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product. The current body of literature supports a role for *MYOC* variants causing JOAG or POAG via a mechanism by which the mutant protein is not secreted extracellularly (Caballero et al., 2000; Gobeil et al., 2004; Izumi et al., 2003; Jacobson et al., 2001; Vollrath

& Liu, 2006), leading to the accumulation of insoluble aggregates in the cells (Caballero & Borrás, 2001; Liu & Vollrath, 2004). The Glaucoma VCEP determined that assays that report on the solubility and secretion of MYOC were suitable, with evidence supporting insolubility and non-secretion of mutant MYOC protein indicating impact on protein function. Transgenic mice expressing the Tyr423-His MYOC mutant (corresponding to human MYOC p.Tyr437His) (Senatorov et al., 2006), or the human p.Tyr437His (Zode et al., 2011), develop elevated intraocular pressure, retinal ganglion cell death and axonal degeneration, reproducing the glaucoma phenotype seen in humans. We determined that animal models that replicate the glaucoma phenotype were also suitable.

Following the recommendations from Brnich et al. (2019), PS3 should only be applied if the assay includes negative (e.g., empty vector) and positive (e.g., wild-type) controls and includes technical and/or biological replicates. The Glaucoma VCEP reviewed all variants included in functional assays that reported on the solubility or the secretion of MYOC (Supp Tables S3a and S3b). We decided to apply different levels of strength based on the odds of pathogenicity (OddsPath) (Brnich et al., 2019) with Strong, Moderate or Supporting levels for OddsPath of >18.7, >4.3, and >2.1, respectively. Validation controls (variants classified as P/LP (pathogenic control) or B/LB (benign control) without the use of PS3 or BS3) from studies that reported on the same class of assay and had the same methodology were combined to calculate the OddsPath.

All coding variants curated in the pilot phase as B/LB for which functional evidence was available were secreted and/or soluble. Similarly, among the coding variants showing evidence for solubility and/or secretion, none were classified as P/LP. Therefore, we decided to apply BS3 (no evidence of damaging effects on protein function in well-established assays) to variants showing solubility or secretion in functional assays that meet the OddsPath of <0.48 or <0.23 for a Supporting or Moderate level, respectively (Brnich et al., 2019). Although there is no evidence currently supporting other protein functions leading to the condition, we recommended not applying BS3 at a Strong level, as we could not completely rule out other mechanisms for pathogenicity.

If multiple results from functional assays are available for a single variant, then the evidence from the assay that is best validated should apply as suggested (Brnich et al., 2019). If results from different assays are conflicting for a single variant, then the result from the assay with the highest level of validation and a conclusive result should override the result from the other assay. However, if the results from the study with the highest level of validation are inconclusive (partial secretion or insoluble levels), then PS3/BS3 should not be applied.

3.5 | Segregation data (PP1_Strong, PP1_Moderate, and PP1)

In the ACMG/AMP guidelines, PP1 applies for co-segregation with disease but is not quantitatively defined. Two studies have since provided quantitative guidelines for assessing segregation of a variant. Jarvik and Browning (2016) calculated a probability that the observed variant and affection status occurs by chance rather than due to co-segregation. Kelly et al. (2018) adopted different numbers of meioses based on likelihood ratios of 10 (LOD 0.9), 30 (LOD 1.5), and 100 (LOD 2.1) for Supporting, Moderate and Strong evidence. The pilot study compared the thresholds for the number of meioses required to apply each level of evidence using both methods. Of the 81 pilot-phase variants, only 2 had a different strength of evidence based on the two approaches: p.Ala363Thr and p.Asn480Lys. The p.Ala363Thr variant had 5 meioses in 3 families, meeting PP1_Moderate according to Kelly et al. but PP1_Strong according to Jarvik and Browning, but both approaches resulted in an LP classification. The p.Asn480Lys (c.1440C>G) variant had 4 meioses in one family that met PP1 and was classified LP according to Kelly et al. but PP1_Moderate with classification of P according to Jarvik and Browning. The Glaucoma VCEP recommends a conservative approach and applied the recommendations from Kelly et al. (2018) with ≥7, ≥5, and ≥3 meioses for Strong, Moderate and Supporting levels. In addition, a Strong level should only be applied if the variant is present in more than one family, to limit the risk of detecting segregation with a second variant in strong linkage disequilibrium with the one under assessment.

Benign variants may segregate in a family by chance or appear to segregate because they are common in the population. The Glaucoma VCEP agreed that PP1 would only apply if BA1 or BS1 were not met. Due to the late age of onset of POAG, the documented reduced penetrance of many variants, and the potential for phenocopies within families, only genotype-positive/phenotype-positive individuals and obligate carriers/phenotype-positive individuals should be counted. Individuals who carry the variant but do not have a diagnosis of JOAG/POAG (genotype-positive/phenotype-negative) should not be included when counting meioses nor should JOAG/POAG patients who do not carry the variant (genotype-negative/phenotype-positive). Phenotype-positive individuals need to have been clinically assessed and either have a diagnosis of glaucoma (POAG or JOAG) or suspicious signs of glaucoma (e.g., ocular hypertension and suspicious discs).

3.6 | De novo data (PS2/PM6_Strong, PS2_Moderate/PM6, and PS2_Supporting/PM6_Supporting)

PS2 and PM6 apply to de novo variants. *MYOC* de novo variants are rare, with only 2 reports in the literature: p.Val251Ala (Kuchtey et al., 2013) and p.Pro254Leu (Souzeau et al., 2016), both confirmed as de novo. The Glaucoma VCEP adopted the SVI-proposed point recommendations for PS2 and PM6 (Figure S2) (Sequence Variant Interpretation Working Group, 2021). Under the scheme, the two criteria are equivalent and only one should be applied. We recommended applying the point-based system for a "phenotype consistent with the gene but not highly specific and with high genetic

heterogeneity" for POAG and a higher point-based system for JOAG, using a "phenotype consistent with gene but not highly specific". Paternity and maternity need to be confirmed to demonstrate a variant is de novo, as per the ACMG/AMP guidelines. Both parents need to be clinically assessed and should not have a diagnosis of glaucoma. If a parent has suspicious signs of glaucoma, their age and the severity of the symptoms should be considered before applying these criteria.

3.7 | Rules removed or determined not applicable

PP5 (reputable source reports as pathogenic) and BP6 (reputable source reports as benign) were removed based on the recommendation from the SVI Working Group to use primary data instead of relying on assertions (Biesecker & Harrison, 2018). The 11 rules determined not applicable in the context of *MYOC* with POAG/JOAG are detailed in Table S1. Of note, PVS1 (truncating variants) was removed as it refers specifically to null variants where loss of function is a known mechanism. *MYOC* variants are known to cause disease through gain-of-function mechanisms and pathogenic truncating variants are not null alleles. These types of variants have been included in the specification for PM4 (protein length changes). The other excluded rules also relate to characteristics of the gene or disease that are not relevant to *MYOC* or POAG/JOAG.

3.8 | Combining criteria

Tavtigian et al. (2018) showed that the ACMG/AMP guidelines were compatible with a Bayesian framework and that they could be converted into a point-based system (Tavtigian et al., 2020). With the point-based system, variants with conflicting evidence can be classified as pathogenic or benign depending on the number and/or the strength of the criteria applied. Additionally, this approach allows the use of criteria strength combinations not specifically listed in the ACMG/AMP guidelines. The Glaucoma VCEP decided to apply the scaled point system recently developed by Tavtigian et al. (2020) (Tables 2 and 3). We modified the threshold for LB from −1 to −2 to follow the ACMG/AMP guidelines that require ≥2 Benign Supporting criteria for an LB classification.

TABLE 2 Point Values for ACMG/AMP strength of evidence categories (Tavtigian et al., 2020)

Evidence strength	Point scale	
	Pathogenic	Benign
Indeterminate	0	0
Supporting	1	−1
Moderate	2	−2
Strong	4	−4
Very strong	8	−8

3.9 | Application of the rules in a pilot study

We curated 81 *MYOC* variants (Table S4) using the specified rules (Table 1). The majority of variants (74%, 60/81) were missense (Figure 1) and were located in exon 3 (78%, 63/81) where the largest numbers of causative variants have been reported.

Figure 2 shows the final classification of the pilot variants. All of the LP and P variants were located in exon 3 (Figure 2b). The B, LB, and VUS variants were located throughout the gene.

Figure 3 shows the distribution of the classification scores for the variants (Table S4). All the variants classified as B met BA1. All the variants with a score of −6 met BS1 as well as BS3_Moderate or a combination of BP4 and BP7. All of the variants classified on the upper end of LB, with a score of −2, solely met BS3_Moderate.

PM2_Supporting was the most applied criterion (Figure 4), used in 59% (48/81) of the classifications including 7 P, 19 LP, and 22 VUS. Because no criteria are applied at a Very Strong level with the specified rules, at least one Strong criterion is required to reach a P classification. PP1_Strong was used in the classification of all P variants along with PS4 (Strong in 5/8 and Moderate in 3/8 variants),

TABLE 3 Point-based variant classification categories, modified from Tavtigian et al. (2020)

Category	Point ranges
Pathogenic	≥10
Likely pathogenic	6–9
Uncertain	−1 to 5
Likely benign	−2 to −6
Benign	≤−7

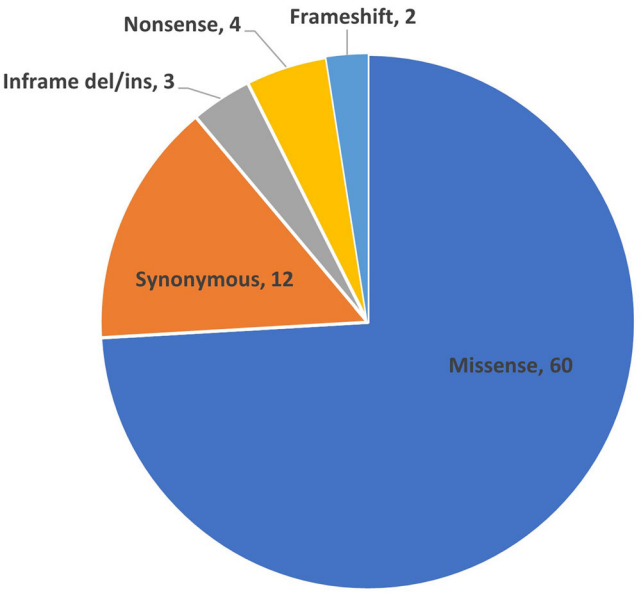

FIGURE 1 Distribution of 81 *MYOC* variants classified in the pilot study.

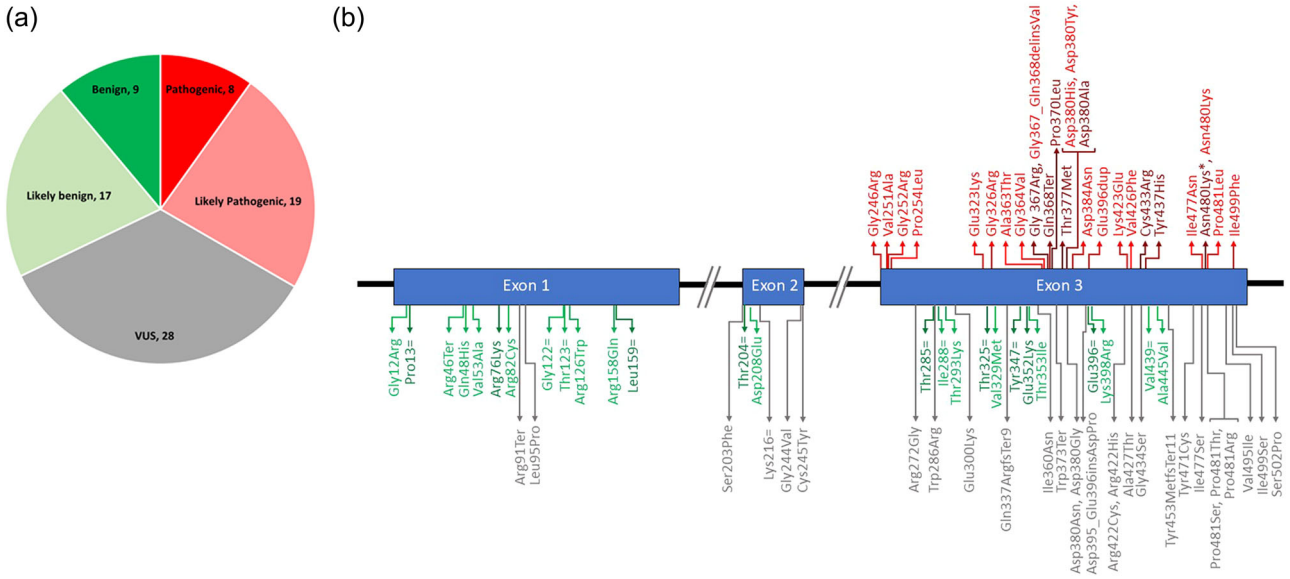

FIGURE 2 Final classification of pilot variants by the Glaucoma VCEP using the specified rules. (a) number of variants in each classification. (b) position of each variant in the *MYOC* gene. Dark red = P; Light red = LP; Grey = VUS; Light green = LB; Dark green = B. *there are 2 variants encoding p.Asn480Lys, c.1440C>A was classified as P and c.1440C>G as LP.

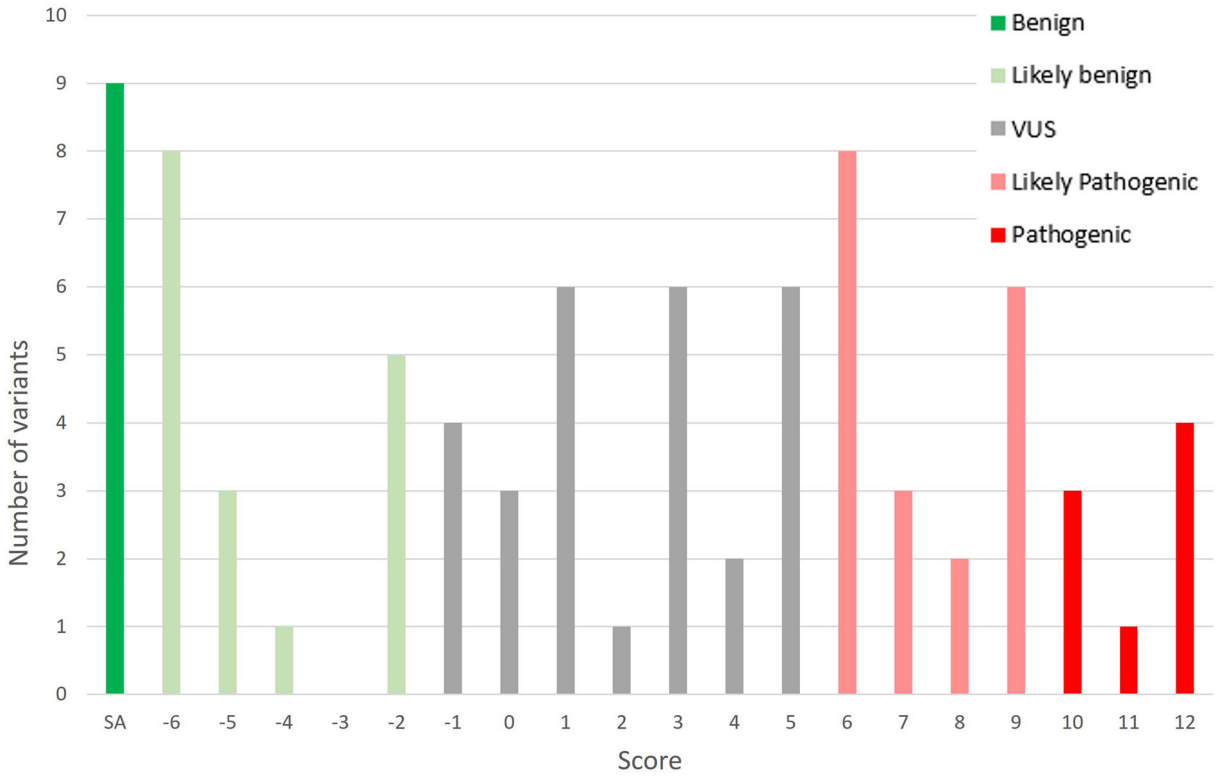

FIGURE 3 Distribution of the final Glaucoma VCEP classification scores.

with PS3_Moderate, PM2_Supporting, and PP3 all included in 7 out of the 8 P classifications.

BA1 was used to classify 9 variants as B. BS1 and BS3_Moderate were the most used criteria for the variants classified as LB (applied 12 and 11 times, respectively). Five variants had conflicting evidence. BS3_Moderate was included in all, as was PM2_Supporting and/or PP3. All of these variants were classified as VUS.

Figure S3 shows that most variants classified as LP or P had PM2_Supporting applied and were not reported in gnomAD, with the exception of the 2 well-characterized common variants p.Gln368Ter

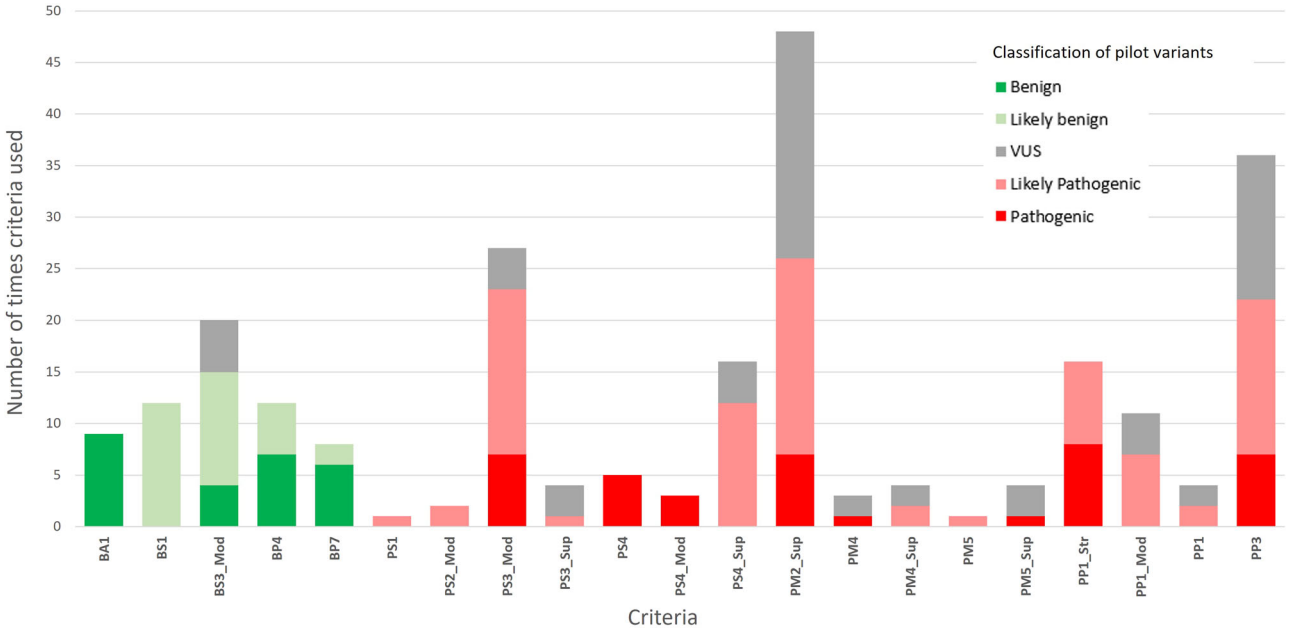

FIGURE 4 Criteria applied in the pilot phase with final Glaucoma VCEP classification of variants.

and p.Thr377Met. It is also evident that BS1 plays an important role in discriminating LB variants from VUS, with 81% (21/26) of variants classified as LB/B meeting BS1 or BA1.

PM5 was used once with an unmodified strength and 4 times as PM5_Supporting. Based on the LP classification of p.Asp380His, PM5_Supporting was applied to p.Asp380Ala, which has a higher Grantham score and was classified as P. This P variant was then used to apply PM5 to p.Asp380Tyr, as it has a higher Grantham score than p.Asp380Ala, increasing its classification to LP. The LP variant p.Asp380His was used to apply PM5_Supporting to p.Asp380Gly, which was classified as VUS. Additionally, PM5_Supporting was applied to p.Pro481Arg and p.Ile499Ser, but these variants also remained as VUS.

The Glaucoma VCEP reviewed evidence from 10 published studies with functional assays, which included 63 variants from the pilot list. BS3_Moderate was applied 20 times in total, with 11 of these involved with LB classifications. For 5 variants, BS3_Moderate was the only criterion applied, which resulted in an LB classification. PS3 was applied 31 times, 27 at the Moderate level and 4 at the Supporting level. Functional evidence influenced the classification for 29% (18/63) of variants, including 10 from VUS to LP, 5 from VUS to LB, and 3 from LP to P.

Among the 40 variants that had a ClinVar classification (accessed January 10, 2022), 40% (16/40) had a change of classification after Glaucoma VCEP curation (Figure 5). Variants with a discordant classification from ClinVar included 7 VUS reclassified as B/LB, 3 LP/P reclassified (2 as VUS and 1 as B/LB) and all 6 variants with conflicting interpretation reclassified (5 as B/LB and 1 as VUS). BA1 and BS1 were applied in the curations of 8 of the 13 variants reclassified as B/LB by the VCEP. BS3_Moderate was applied to 10 of these variants. The number of variants in ClinVar classified as VUS or as variants with conflicting interpretations decreased from 13 to 1 after VCEP curation.

4 | DISCUSSION

The specifications of the ACMG/AMP guidelines for the *MYOC* gene in relation to POAG/JOAG has resulted in the reclassification of over one-third of the variants reported in ClinVar. The process has led to fewer VUS, with clearer definitions of LB and B, largely related to population frequency and functional evidence. Similarly, for LP and P classifications, population and functional criteria were influential, but in contrast to the benign variants, in silico predictions were frequently applied and influenced the classification.

Challenges were revealed where information from previously classified variants was required to classify a variant of interest. This was evident in the application of PS1, PM5, and PS3/BS3. Curators were first required to identify variants in our pilot list that met the LP or P classifications without the application of these rules before we could apply these rules to other variants. For example, PM5_Supporting or PM5 can only be applied if another variant at the same residue was previously classified as LP or P and the novel variant must have a higher Grantham score than the previously classified variant. This means biocurators were required to seek information about many other variants to apply the rule to one variant. Similarly, for PS3 and BS3, it was necessary to count the number of variants assessed by that assay that were classified B/LB or LP/P without functional evidence, and label these as validation controls for that assay to determine the strength at which each rule can be applied. We expanded the pilot variant list to include all variants with functional evaluation in the literature to facilitate this process. Generating

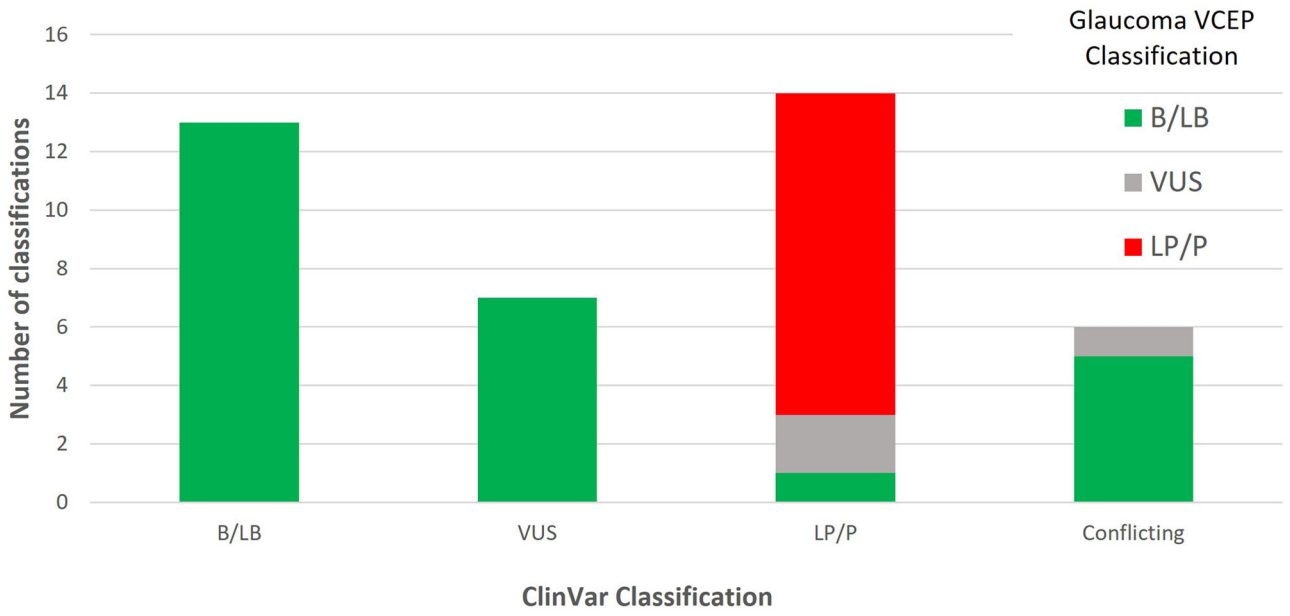

FIGURE 5 ClinVar versus Glaucoma VCEP classification of *MYOC* variants from the pilot phase. Colors indicate the Glaucoma VCEP classification.

functional evidence for additional variants beyond the pilot list will be highly valuable, especially for variants classified as VUS in the absence of functional data.

The addition of carefully assessed functional data to the classification resulted in 15 VUS now meeting the thresholds for LB or LP. We took a conservative approach, carefully evaluating the strength of each assay, guided by the work by Brnich et al. (2019) to quantify the required numbers of validation controls. Even with the conservative evidence-based thresholds, these criteria played an important role in the classification. The VCEP chose to specify the application of BS3 at the Moderate level when the appropriate OddsPath was reached, as described by Brnich et al. (2019). This is in contrast to the original ACMG/AMP guidelines, which did not allow for any benign criteria to be applied at the Moderate level. We do not recommend applying BS3 at the Strong level. With uncertainty remaining as to whether the solubility and secretion assays test all the appropriate or possible disease mechanisms, the VCEP took the conservative approach of not overclassifying variants towards benign. As knowledge of POAG molecular mechanisms increases and more thorough assays are developed, the specifications for this criterion may need to be re-assessed. The application of BS3_Moderate allowed variants to be classified as LB with the application of BS3_Moderate alone (score of −2). This decision was taken to reflect a consistent approach in the level of evidence required for both BS3 and PS3 and reflects the level of confidence the Glaucoma VCEP had in the quality and validity of the functional assays described for MYOC.

Despite PP1 in the original guidelines being at the Supporting level, this rule is often applied arbitrarily at higher levels. The work by Kelly et al. (2018) in relation to the *MYH7* gene, and Jarvik and Browning (2016) for more general application, provide an evidence base for rational decision-making on when to apply each level of evidence. Both are simple to apply, requiring only the counting of meioses with thresholds for each level of significance correlating to the probabilities of multiple co-segregating transmissions. We chose the slightly more conservative classifications of Kelly et al. (2018) to limit over-interpretation; however, the marginal differences in outcome reflect the similarity in the underlying approaches. PS4 was originally defined for case-control studies, or, when they do not reach statistical significance, for multiple independent affected individuals. Similar to PP1, we have developed an easy-to-use points system that allows counting of independent probands and the application of PS4 at different levels of evidence.

The standard benign criteria for population data are BA1 (allele frequency >0.05) and BS1 (allele frequency greater than expected for the disease). However, it has been recognized that the prevalence and penetrance of the disease as well as the gene contribution should be considered when applying these rules (Whiffin et al., 2017). The Glaucoma VCEP developed allele frequency thresholds for BA1 and BS1 that reflect the architecture of the disease and the *MYOC* gene. Our pilot data show that 81% of LB/B variants met one of these criteria, validating our approach. Similarly, PM2 was initially defined as an absence of the variant from controls. We set a conservative threshold to account for the possibility of pathogenic variants being present in population databases in the context of incomplete age-related penetrance and undiagnosed glaucoma in the population. Nevertheless, all but 2 of the variants classified as LP/P from the pilot study were absent from gnomAD, highlighting the rarity of pathogenic *MYOC* variants in population databases. Although we used gnomAD version 2 for assessing allele frequency in the pilot phase, we encourage the use of other large population datasets that become available or that may be specific to some populations.

The Glaucoma VCEP accessed allele frequency information from gnomAD and recommended applying the most relevant population-specific information where available. It should be noted that gnomAD and most other population databases are limited in their content from non-European populations. Our pilot variant list included several variants predominantly reported in non-European probands. Several were classified as B (p.Pro13Pro seen in African/African American) or LB (p.Gly12Arg, p.Arg46Ter, p.Gln48His, p.Asp208Glu, p.Thr353Ile reported in East Asian/South Asian). Others were classified as VUS (p.Trp286Arg in Latino/Admixed American and p.Glu300Lys in East Asian) and 2 as P (p.Thr377Met and p.Cys433Arg - African/African American). As access to genetic research and testing increases in populations of non-European descent, sourcing appropriate population frequencies will be critically important for correctly interpreting novel variants. New information from other populations should also be considered for variants already classified that may have inadvertently been labeled rare based on European information but are more common in other populations.

Access to high-quality data is important for criteria requiring counting of genotype- and phenotype-positive individuals (PS4, PP1). In the pilot study, this was largely achieved through manual curation of published literature, including, where necessary, contacting the corresponding authors to confirm overlapping individuals between publications. Where this information could not be obtained directly, we took the conservative approach of only counting each possibly duplicated patient once. In addition, the Glaucoma VCEP accessed multiple research databases, increasing the number of probands counted for some variants. This highlights the importance of data sharing through individual research groups and accredited genetic-testing laboratories publishing and/or depositing the information for each observed variant in publicly accessible locations. This is especially important for rarely observed variants where every piece of information can have a significant effect on the overall classification.

The thresholds for each criterion are in line with those recommended by other VCEPs for similar autosomal dominant heterogeneous diseases such as *MYH7*-associated inherited cardiomyopathies (Kelly et al., 2018), genetic hearing loss (Oza et al., 2018) and myeloid malignancy caused by *RUNX1* variants (Luo et al., 2019). This consistency in approach provides a framework that can be applied to other diseases and genes that have similar characteristics but have not yet had the benefit of dedicated VCEP review and rule specification. This is important, as the workload for defining specific rules for every disease-causing gene is daunting. The genetic hearing loss VCEP has specified a set of rules to be applied across a range of genetic hearing loss-related genes (Oza et al., 2018), streamlining the process for this group of genes that all have similar characteristics. This approach will be necessary for the efficient specification of rules for other heterogeneous but monogenic diseases.

The Glaucoma VCEP has curated 81 variants using *MYOC*-specific rules within the framework of the ACMG/AMP guidelines and these classifications are available in ClinVar. The VCEP will curate the remaining reported variants and novel variants as they are reported. We will also review any variants submitted to ClinVar with a different classification to that assigned by the VCEP, as additional evidence may change the classifications. Variants with a medically significant difference (P/LP vs B/LB/VUS) will be reassessed within 3 months of being notified of the discrepant ClinVar classification. Variants classified as LP and VUS will be reviewed every 2 years and LB variants will be reassessed when new large population datasets are released as per ClinGen protocol, to ensure up-to-date information is available for all variants. The VCEP will review its *MYOC*-specified rules every 2 years or sooner as necessary if new knowledge or recommendations from ClinGen arise.

ACKNOWLEDGMENTS

This study was funded by a National Health and Medical Research Council (NHMRC) of Australia Centre for Research Excellence grant (GNT1116360), Program Grant (GNT1150144), Practitioner Fellowships to J.E.C, D.A.M and A.W.H., The Hospital Research Foundation Early Career Fellowship to E.S, A Core Grant for Vision Research from the National Eye Institute/National Institutes of Health to the University of Wisconsin-Madison (P30EY016665) and an Unrestricted Grant from Research to Prevent Blindness, Inc. to the UW-Madison Department of Ophthalmology and Visual Sciences to T.L.Y. and K.N.W. The authors would like to acknowledge the support of the ClinGen Sequence Variant Interpretation and Ocular Clinical Domain Working Groups, especially Kristy Lee.

WEB RESOURCES

Whiffin/Ware calculator: https://cardiodb.org/allelefrequencyapp/
Glaucoma VCEP: https://www.clinicalgenome.org/affiliation/50053/
REVEL score: https://sites.google.com/site/revelgenomics/downloads
CADD score (v1.6): https://cadd.gs.washington.edu/snv
SpliceAI score: https://spliceailookup.broadinstitute.org/
GERP score: https://genome.ucsc.edu/cgi-bin/hgGateway
ClinGen: https://clinicalgenome.org/
ClinGen Variant Curation Interface: https://curation.clinicalgenome.org/
ClinVar: https://www.ncbi.nlm.nih.gov/clinvar/
gnomAD: https://gnomad.broadinstitute.org/
Myocilin database: http://www.myocilin.com

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ORCID

Kathryn P. Burdon http://orcid.org/0000-0001-8217-1249
John D. Hulleman http://orcid.org/0000-0001-8149-656X
Emmanuelle Souzeau http://orcid.org/0000-0002-2015-6577

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How to cite this article: Burdon, K. P., Graham, P., Hadler, J., Hulleman, J. D., Pasutto, F., Boese, E. A., Craig, J. E., Fingert, J. H., Hewitt, A. W., Siggs, O. M., Whisenhunt, K., Young, T. L., Mackey, D. A., Dubowsky, A., & Souzeau, E. (2022). Specifications of the ACMG/AMP variant curation guidelines for myocilin: Recommendations from the clingen glaucoma expert panel. *Human Mutation*, 43, 2170–2186. https://doi.org/10.1002/humu.24482