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Authors

Parsons, Michael T

de la Hoya, Miguel

Richardson, Marcy E

et al.

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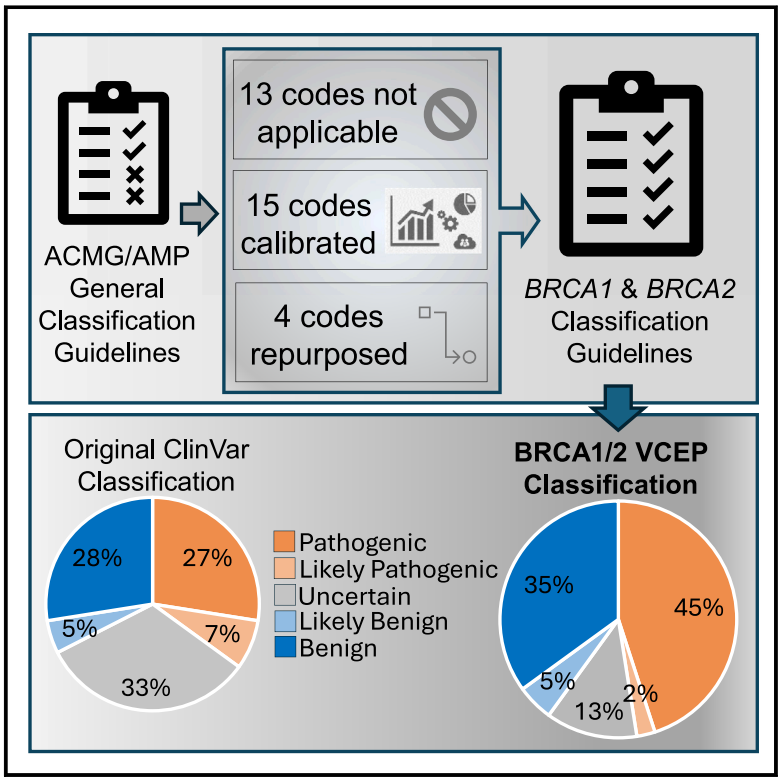
Graphical abstract



Authors

Michael T. Parsons, Miguel de la Hoya, Marcy E. Richardson, ..., Maaike P.G. Vreeswijk, David E. Goldgar, Amanda B. Spurdle

Correspondence

michael.parsons@qimrberghofer.edu.au (M.T.P.), amanda.spurdle@qimrberghofer.edu.au (A.B.S.)

This work presents an overview of gene-specific protocols for assessing the clinical relevance of sequence changes in the BRCA1 and BRCA2 breast cancer risk genes and their value for resolving clinical certainty after gene testing. These publicly accessible protocols can now be used for improved genetic diagnosis and thereby patient management.

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ARTICLE

Evidence-based recommendations for gene-specific ACMG/AMP variant classification from the ClinGen ENIGMA BRCA1 and BRCA2 Variant Curation Expert Panel

Michael T. Parsons,[1,*] Miguel de la Hoya,[2] Marcy E. Richardson,[3] Emma Tudini,[1] Michael Anderson,[4] Windy Berkofsky-Fessler,[5] Sandrine M. Caputo,[6] Raymond C. Chan,[7] Melissa S. Cline,[8] Bing-Jian Feng,[9] Cristina Fortuno,[1] Encarna Gomez-Garcia,[10] Johanna Hadler,[1] Susan Hiraki,[5] Megan Holdren,[11] Claude Houdayer,[12] Kathleen Hruska,[5] Paul James,[13] Rachid Karam,[3] Huei San Leong,[14] Alexandra Martins,[15] Arjen R. Mensenkamp,[16] Alvaro N. Monteiro,[17] Vaishnavi Nathan,[1] Robert O'Connor,[7] Inge Sokilde Pedersen,[18,19,20] Tina Pesaran,[3] Paolo Radice,[21] Gunnar Schmidt,[22] Melissa Southey,[23,24,25] Sean Tavtigian,[26] Bryony A. Thompson,[27] Amanda E. Toland,[28] Clare Turnbull,[29] Maartje J. Vogel,[30] Jamie Weyandt,[3] George A.R. Wiggins,[31] Lauren Zec,[32] Fergus J. Couch,[11] Logan C. Walker,[31] Maaike P.G. Vreeswijk,[33] David E. Goldgar,[9] and Amanda B. Spurdle[1,34,*]

Summary

The ENIGMA research consortium develops and applies methods to determine clinical significance of variants in hereditary breast and ovarian cancer genes. An ENIGMA *BRCA1/2* classification sub-group, formed in 2015 as a ClinGen external expert panel, evolved into a ClinGen internal Variant Curation Expert Panel (VCEP) to align with Food and Drug Administration recognized processes for ClinVar contributions.

The VCEP reviewed American College of Medical Genetics and Genomics/Association of Molecular Pathology (ACMG/AMP) classification criteria for relevance to interpreting *BRCA1* and *BRCA2* variants. Statistical methods were used to calibrate evidence strength for different data types. Pilot specifications were tested on 40 variants and documentation revised for clarity and ease of use.

The original criterion descriptions for 13 evidence codes were considered non-applicable or overlapping with other criteria. Scenario of use was extended or re-purposed for eight codes. Extensive analysis and/or data review informed specification descriptions and weights for all codes. Specifications were applied to pilot variants with pre-existing ClinVar classification as follows: 13 uncertain significance or conflicting, 14 pathogenic and/or likely pathogenic, and 13 benign and/or likely benign. Review resolved classification for 11/13 uncertain significance or conflicting variants and retained or improved confidence in classification for the remaining variants.

Alignment of pre-existing ENIGMA research classification processes with ACMG/AMP classification guidelines highlighted several gaps in the research processes and the baseline ACMG/AMP criteria. Calibration of evidence strength was key to justify utility and strength of different data types for gene-specific application. The gene-specific criteria demonstrated value for improving ACMG/AMP-aligned classification of *BRCA1* and *BRCA2* variants.

[1]Population Health, QIMR Berghofer Medical Research Institute, Brisbane, QLD 4006, Australia; [2]Molecular Oncology Laboratory, Hospital Clínico San Carlos, IdISSC, 28040 Madrid Spain; [3]Ambry Genetics Corporation, Aliso Viejo, CA 92656, USA; [4]Invitae Corporation, San Francisco, CA, USA; [5]GeneDx, Gaithersburg, MD, USA; [6]Department of Genetics, Institut Curie, and Paris Sciences Lettres Research University, 75005 Paris, France; [7]Color Health, Burlingame, CA, USA; [8]UC Santa Cruz Genomics Institute, Genomics, University of California, 1156 High Street, Santa Cruz, CA 95064, USA; [9]Department of Dermatology, Huntsman Cancer Institute, University of Utah School of Medicine, Salt Lake City, UT, USA; [10]Department of Clinical Genetics, Maastricht University Medical Center, Maastricht, the Netherlands; [11]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA; [12]University Rouen Normandie, Inserm U1245 and CHU Rouen, Department of Genetics, FHU G4 Génomique, F-76000 Rouen, France; [13]Sir Peter MacCallum Department of Oncology, The University of Melbourne, Melbourne, VIC, Australia; [14]Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, VIC 3052, Australia; [15]University Rouen Normandie, Inserm U1245, F-76000 Rouen, France; [16]Department of Human Genetics, Radboud University Medical Center, Nijmegen, the Netherlands; [17]Department of Cancer Epidemiology, H Lee Moffitt Cancer Center & Research Institute, Tampa, FL, USA; [18]Molecular Diagnostics, Aalborg University Hospital, Aalborg, Denmark; [19]Department of Clinical Medicine, Aalborg University, Aalborg, Denmark; [20]Clinical Cancer Research Center, Aalborg University Hospital, Aalborg, Denmark; [21]Predictive Medicine: Molecular Bases of Genetic Risk, Department of Experimental Oncology, Fondazione IRCCS Istituto Nazionale Dei Tumori, Via Venezian 1, 20133 Milano, Italy; [22]Institute of Human Genetics, Hannover Medical School, 30625 Hannover, Germany; [23]Cancer Epidemiology Division, Cancer Council Victoria, Melbourne, VIC, Australia; [24]Precision Medicine, School of Clinical Sciences at Monash Health, Monash University, Clayton, VIC 3168, Australia; [25]Department of Clinical Pathology, The Melbourne Medical School, The University of Melbourne, Melbourne, VIC 3010, Australia; [26]Department of Oncological Sciences and Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84132, USA; [27]Department of Pathology, Royal Melbourne Hospital, Melbourne, VIC 3050, Australia; [28]Department of Cancer Biology and Genetics, The Ohio State University College of Medicine, Columbus, OH 43210, USA; [29]Translational Genetics Team, Division of Genetics and Epidemiology, Institute of Cancer Research, London, UK; [30]Department of Human Genetics, Amsterdam University Medical Centers, Amsterdam, the Netherlands; [31]Department of Pathology and Biomedical Science, University of Otago, Christchurch, New Zealand; [32]Natera, Inc, San Carlos, CA, USA; [33]Department of Human Genetics, Leiden University Medical Center, Leiden, the Netherlands; [34]Faculty of Medicine, University of Queensland, Brisbane, QLD, Australia

*Correspondence: michael.parsons@qimrberghofer.edu.au (M.T.P.), amanda.spurdle@qimrberghofer.edu.au (A.B.S.)

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Introduction

The role of *BRCA1* (MIM: 113705) and *BRCA2* (MIM: 600185) in hereditary breast and ovarian cancer (HBOC) has long been recognized with genetic testing initiated soon after discovery of these genes in the 1990s.[1,2] The ENIGMA international research consortium (https://enigmaconsortium.org/)[3] focuses on development and application of methods to determine the clinical significance of sequence variants in HBOC genes. The consortium has members from six continents that provide a broad range of expertise under the umbrellas of analytical, splicing, functional, pathology, and clinical working groups for translational research projects. At the request of ClinGen, in 2015 ENIGMA formed an external expert panel for curation of *BRCA1* and *BRCA2* variants. The classification criteria documented for this purpose captured qualitative criteria, generally adopted clinically (e.g., most premature termination codon variants were assumed to be pathogenic), and quantitative multifactorial likelihood analysis methods developed in the research setting.[4–8] The key component of the multifactorial likelihood approach is the statistical calibration of independent data types using variants of known pathogenicity status to inform the weight of evidence toward or against pathogenicity. The external expert panel guidelines were then used to assign 7,456 expert curations for *BRCA1* and *BRCA2* variants in ClinVar.

In parallel to these efforts, there was increasing international uptake of variant classification guidelines published by the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP)[9] for diagnostic interpretation of germline sequence variants with applicability to any Mendelian disease. In 2020, the ENIGMA external expert panel sought to become an internal ClinGen Variant Curation Expert Panel (VCEP),[10,11] to align with Food and Drug Administration (FDA) recognized processes for expert panel contributions to ClinVar. Here, we provide an overview of the evidence-based approach taken to consider relevance of each ACMG/AMP evidence code for curation of variants in *BRCA1* and *BRCA2* and report pilot study results, demonstrating the value of detailed (gene-specific) specifications to assist variant curation and resolve discordances and uncertainty in variant classification.

Methods

The establishment and activities of the ClinGen BRCA1 and BRCA2 VCEP followed the ClinGen FDA-recognized approval process (https://clinicalgenome.org/docs/guidelines-for-applying-for-variant-or-gene-curation-expert-panel-status/), with reference to Protocol version 8 at the time of VCEP initiation.

The study curated already de-identified data from individuals with variants and aggregate information, which did not include individual-level data or any protected health information. As such, it does not constitute human subject research.

The original ENIGMA BRCA1 and BRCA2 expert panel membership, which was largely comprised of representatives from major national clinical and research initiatives in Australia, Europe, and the United States, was expanded to include representatives from several major diagnostic testing laboratories from the United States with extensive experience in the application of ACMG/AMP guidelines. The resulting ClinGen ENIGMA BRCA1 and BRCA2 VCEP consists of research and clinical experts currently spanning representation from Australasia, Europe, and the United States. VCEP members met approximately monthly to review the baseline ACMG/AMP sequence variant classification guidelines[9] to determine whether each classification criterion should be adopted, modified, or omitted for *BRCA1* and *BRCA2* variant interpretation.

Both *BRCA1* and *BRCA2* were designated as genes for which loss of function is a known mechanism of disease. Reference sequences used for annotation are as follows:

BRCA1: coding DNA reference sequence from genomic RefSeq NG_005905.2 (same as LRG_292, Ensembl ENSG00000012048) covering *BRCA1* transcript GenBank: NM_007294.4 (Ensembl transcript ENST00000357654.9). Exons are sequentially numbered to match the exon descriptions of the MANE Select transcript (GenBank: NM_007294.4). Exon numbering of *BRCA1* has historically been according to GenBank U14680.1 with exon 4 missing due to a correction made after the initial description of the gene, termed here as legacy exon numbering.

BRCA2: coding DNA reference sequence from genomic RefSeq NG_012772.3 (same as LRG_293, Ensembl ENSG00000139618), covering *BRCA2* transcript GenBank: NM_000059.4 (MANE Select transcript; Ensembl transcript ENST00000380152.8).

The classification tiers in pre-existing ENIGMA external panel classification criteria for *BRCA1* and *BRCA2* (no longer in use, Data S1) grouped multiple sources of information (e.g., frequency data, variant type, tumor pathology, co-occurrence with a pathogenic variant). A critical aspect of VCEP activities was to convert these grouped criteria to align with ACMG/AMP codes representing different classification criteria, falling under the broad evidence types described for the ACMG/AMP framework (i.e., population, computation/predictive, functional, segregation, *de novo*, allelic, other).[9] Where possible, statistical methods were used to calibrate strength of evidence (i.e., supporting, moderate, strong, very strong, and stand-alone) for different data types. Statistical methods used to inform or directly weight evidence types included a combination of logistic regression analysis, heterogeneity analysis and likelihood ratio (LR) estimation (as exemplified in a previous ENIGMA publication focused on *BRCA2* exon 3 variants),[12] and a maximum likelihood estimate approach.[13] LR estimates toward or against pathogenicity were derived for a given evidence type using defined reference sets of benign and pathogenic variants (see example calculation in the results section), as exemplified for frequency and functional data in a previous ENIGMA publication,[14] using the statistical method as detailed previously.[15] The LR estimates were then used to assign weights for or against pathogenicity following recommendations arising from Bayesian modeling of the ACMG/AMP guidelines.[16] Population frequency cut-offs for frequency codes were additionally informed by use of the ClinGen recommended calculator (http://cardiodb.org/allelefrequencyapp/) and assumptions relevant for *BRCA1* and *BRCA2* prevalence and penetrance.[17] Further details of the analytical approaches and datasets used and justifications for code applicability and weighting, as approved by the ClinGen

SVI, are available via the ClinGen Specifications registry (https://cspec.genome.network/cspec/ui/svi/affiliation/50087), duplicated here as Data S2. Note that the figure and table numbering for the specifications information included within Data S2 is as approved by the ClinGen SVI and is distinct from the numbering used in this publication.

For ease of reference, we provide methodological details for evidence codes highlighted within this publication by presentation in tabular or figure format. Note S1 provides further details regarding extended calibration analysis for missense variant prediction that is highlighted in this publication: computational prediction of missense variants undertaken to include an uninformative bioinformatic score range category and specifically to compare BayesDel score categories to those recommended for general use by Pejaver et al.,[18] published during the VCEP specification process. Note S2 provides details relating to derivation of per-exon points and weights assigned for protein termination codon (PTC) variants (excluding those resulting from mRNA splicing alterations) under PM5 (PTC). Further information about the data sources and values informing the evidence for each exon is provided in Table S1.

Alongside, key members of the ClinGen Sequence Variant Interpretation Working Group (SVI WG) (https://clinicalgenome.org/working-groups/sequence-variant-interpretation/) were consulted about how to capture valuable information sources and analytical approaches that were used previously for external expert panel classification (duplicated here as Data S1) but that did not strictly conform to ACMG/AMP evidence types and designated codes/strengths. This included how to capture information based on multifactorial likelihood ratio data, where some but not all the evidence types exist as ACMG/AMP codes. The final recommendations arising from SVI review include all previously published multifactorial data under PP4 (or BP5), include new tumor pathology data together with existing multifactorial data under PP4 (or BP5), and describe new segregation data under PP1 (or BS4). Extensive documentation supporting the rationale for application and weighting of each code was compiled for ClinGen SVI WG review following the standard VCEP approval protocol. Specifications for codes relating to the use of computational and experimental evidence relevant to variant impact on RNA splicing were informed by parallel development of recommendations from the ClinGen SVI Splicing Subgroup.[19] After addressing feedback from the SVI WG, the draft documentation was provided to nine VCEP members who had self-nominated to act as biocurators. As biocurators they review and evaluate evidence relevant for variant classification, assign relevant ACMG/AMP codes and weights for the available evidence, and ascribe a final pathogenicity classification based on the information reviewed. The draft specifications were tested on 40 pilot variants, selected to capture variants spanning different assumed molecular impact, and different pre-existing classifications in ClinVar (Table S2). VCEP members were requested to provide any internal data of relevance for classification of these 40 variants. Initial ClinVar summary classification descriptions were as follows: pathogenic (P), $n = 11$; likely pathogenic/pathogenic (LP/P), $n = 3$; uncertain significance (VUS), $n = 4$; benign/likely benign (B/LB), $n = 1$; benign (B), $n = 12$; conflicting, $n = 9$. Conflicting classifications represented various combinations of individual submitter classifications (details provided in Table S2).

To facilitate the curation process, each biocurator was provided a file with the following variant-specific information: population frequency as reported in gnomAD (v2.1 exomes only and v3.1), existing multifactorial LR data (spanning segregation, family history, tumor pathology, case control, and co-occurrence LRs), protein functional assay data, mRNA splicing assay data, bioinformatic impact predictions (missense, in-frame, splicing), clinical features of individuals with Fanconi anemia (FA) as drawn from the literature, and additional internal laboratory data relevant for classification as provided by VCEP members. The latter included splicing assay results, co-segregation data and presence or absence of FA phenotype for individuals with co-occurring variants. Protein functional assay data were provided with functional category (impact, no impact, or partial/indeterminate) assigned for all functional assays considered relevant, with a summary description of the combined results. RNA assay data required evaluation by individual biocurators to assign code weights. Each variant was curated by the lead biocurator (MTP) and two additional biocurators. The lead biocurator reviewed curations for consistency in code application (including code strength) and final variant classification. Collated findings were discussed with all VCEP members to identify factors contributing to between-biocurator differences in use of the specifications.

After this initial phase of variant review, biocurator feedback was used to inform revision of the documentation for clarity and ease of use. This included development of simplified look-up tables. At this time, codes relating to bioinformatic predictions were updated to allow three categories: evidence toward pathogenicity, against pathogenicity, and no bioinformatic code applicable. These updates were based on results from published splicing prediction analyses[19] and VCEP-specific re-analysis conducted to refine calibrations for missense prediction (see above; using approaches as detailed in documentation available via the ClinGen Specifications registry and also provided in Note S1).

The revised documentation was then used for a second phase of the pilot curation. Variants classified with inter-biocurator differences including at least one VUS and one non-VUS classification, labeled as "VUS/other," were reviewed by two additional VCEP biocurators. Variants with classification confidence differences (P versus LP or B versus LB) were reviewed by one independent biocurator with extensive experience from the ClinGen TP53 VCEP. Finally, code assignment was checked for all variants with concordant classification from the first pilot phase by two VCEP biocurators. Further minor revisions were introduced following biocurator feedback on the revised documentation and after final review from the ClinGen SVI WG. Final codes applied and evidence summary are publicly available in the ClinGen Evidence Repository (https://erepo.clinicalgenome.org/evrepo/) and ClinVar database[20] (https://www.ncbi.nlm.nih.gov/clinvar/) (Table S2).

Results and discussion

An overview of the migration of the ENIGMA external expert panel to current operation as a ClinGen-approved VCEP, following FDA-recognized processes, is shown in Figure 1. Development and documentation of the specifications was an iterative process that involved (1) discussions and/or review at multiple levels (within the ClinGen SVI WG and the VCEP members), (2) coordination with and consideration of other ClinGen activities—including other ClinGen hereditary cancer domain VCEPs, the ClinGen SVI Splicing Subgroup,[19] and a subgroup of the ClinGen SVI WG focused on calibration of computational tools for

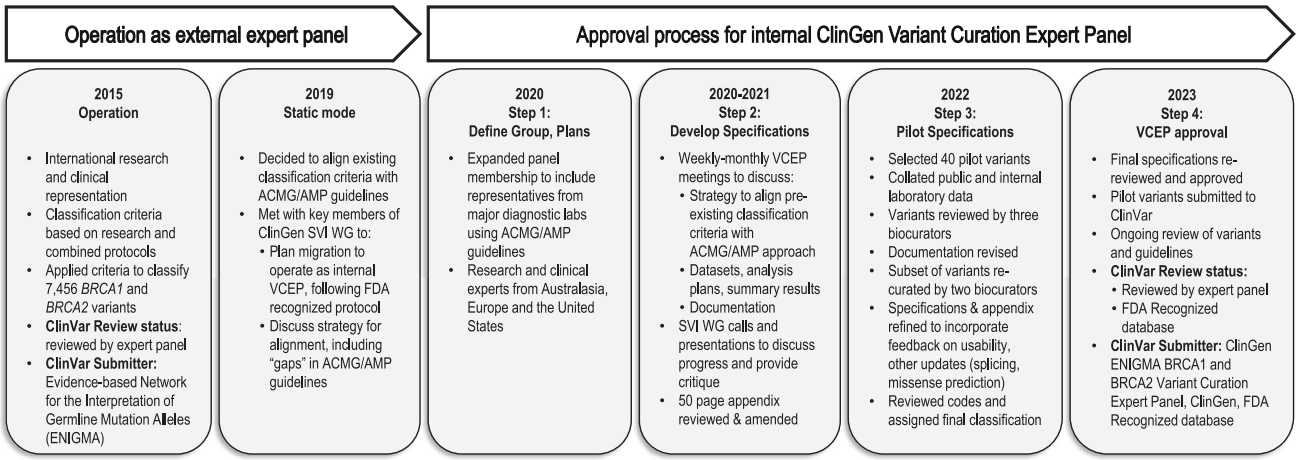

Figure 1. Timeline for migration from "external" expert panel to operation as a ClinGen Variant Curation Expert Panel following the ClinGen FDA-recognized protocol

Discussions with key members of the SVI WG during 2019 relating to the strategy for alignment included the need to capture information sources used previously for *BRCA1* and *BRCA2* variant interpretation that are not explicitly designated under the baseline ACMG/AMP guidelines, especially information used in the context of multifactorial likelihood modeling. Revision of specifications at step 3 included updates in response to ClinGen SVI Splicing subgroup recommendations[19] and re-calibration of bioinformatic prediction of missense impact in response to ClinGen SVI Computational subgroup recommendations.[18] Details of ENIGMA *BRCA1* and *BRCA2* Variant Curation Expert Panel membership and biocurator workforce are available at https://clinicalgenome.org/affiliation/50087/. Pre-existing ENIGMA expert panel classification guidelines for *BRCA1* and *BRCA2* are shown in Data S1 as a point of reference (though no longer currently used). The final specifications first approved for internal ClinGen *BRCA1/2* VCEP use are available via https://cspec.genome.network/cspec/ui/svi/affiliation/50087 and have been duplicated here as Data S2.

missense prediction.[18] The extended timeline reflects the evidence-based approach taken to justify—to both VCEP and ClinGen SVI WG members—the appropriateness and/or strength of different information sources for application to *BRCA1* and *BRCA2* variants.

Overview of *BRCA1* and *BRCA2* specifications

A summary of the specifications designated for each ACMG/AMP code is described in Table 1, together with a brief description of mode of application or reasons for excluding a given code. These specifications are expected to be updated over time to follow on scientific knowledge progress, and version changes will be documented via the ClinGen Criteria Specification Registry (https://cspec.genome.network/cspec/ui/svi/affiliation/50087). Summary findings reported in this study refer to version 1.0 specifications.

After initial review of ACMG/AMP criteria for relevance to interpretation of *BRCA1* and *BRCA2* variants, the original criterion descriptions for 13 codes were considered non-applicable to these genes or overlapping (non-independent) with other criteria largely based on expert opinion (Table 1). Specific examples were PS2/PM6 (*de novo*), given that *BRCA1/2*-related cancers are common, and there was no information available to calibrate use of this information type and PM1 (location in a hot spot or critical domain), since this was considered to be captured as a component of bioinformatic analysis as missense prediction tools inherently capture this information. In addition, directed calibration analysis was undertaken to justify that generalized use of proband counting as PS4_Moderate is inappropriate for these genes due to overlap with frequency codes and variability in evidence strengths observed between cohorts.[21]

Specifications were denoted for 15 codes. Extensive data review and/or analysis from previous ENIGMA-wide and/or VCEP activities was used to inform processes and relevant weights applicable for most of these 15 codes, as described in comprehensive supplementary documentation provided with the VCEP specifications, captured as v1.0 in the ClinGen online registry for VCEP specifications (https://cspec.genome.network/cspec/ui/svi/affiliation/50087; all versions of specifications are made available via this resource). Several specifications were implemented specifically to follow recommendations from parallel work of the ClinGen SVI Splicing Subgroup[19]: codes PVS1, PS1, and BP7 were extended to capture RNA splicing experimental data or *in silico* predictions; PS3 and BS3 were restricted to capture results from assays that measure protein functional effect (either only protein impact, or protein impact that also measures underlying mRNA impact[s]); and splicing impact thresholds defined for SpliceAI were set for bioinformatic prediction of variant impact on splicing, captured under various codes. Probability analysis combined with LR estimation[12] had been used to select and weight bioinformatic tool score categories for missense variant prediction under PP3 and BP4. BayesDel[22] was selected as tool of choice based on results from heterogeneity analysis, performance compared to similar tools, and ability to provide scores for in-frame indels. Extended calibration analysis was undertaken during the pilot phase (Note S1) to include an uninformative bioinformatic score

Table 1. General description of BRCA1 and BRCA2 VCEP specifications for ACMG/AMP baseline criteria and evidence codes

Evidence code[a]	Simplified criterion description[a]	Included in BRCA1 and BRCA2 specifications	Extension and other comments	Relevant appendix sections, and specifications figures and tables in ClinGen Specification Registry, with numbering as for original approved specifications[b]
Pathogenic criteria				
PVS1	null variant in a gene where loss of function is a known mechanism of disease	yes, variable weight	detailed PVS1 flowcharts for protein termination codon (PTC) and splice site donor and acceptor +/−1,2 dinucleotide variants that account for knowledge of naturally occurring transcripts	Appendix E and J; Specifications Figure 5
		additional use to capture splicing	variant-induced transcripts interpreted via the PVS1 decision tree	
PS1	same amino acid change as a previously established pathogenic variant regardless of nucleotide change	yes, variable weight	apply for same missense change caused by a different nucleotide change	Appendix E and J; Specifications Figure 5
		additional use to capture splicing	apply PS1_Variable Weight, for exonic and intronic variants with same predicted impact on splicing, as a previously classified (likely) pathogenic splicing variant; vary weight depending on relative positions and confidence in classification of the reference variant	
PS2	*de novo* (paternity confirmed) in a patient with the disease and no family history	no	*BRCA1/2*-related cancers occur relatively commonly; no information to calibrate the predictive capacity of *de novo* occurrences	–
PS3	well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect	yes	assay measures effect via protein only OR mRNA and protein combined; splicing-only assay data captured under other PVS1_Variable (RNA)	Appendix E; Specifications Figure 1C
PS4	the prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls	yes	case-control LR is the preferred approach; otherwise, case-control p value ≤ 0.05 and OR ≥ 4 (lower confidence interval excludes 2.0)	Appendix F
PS4_Moderate	proband counting: prior observation of the variant in multiple unrelated patients with the same phenotype and its absence in controls	no	proposed in Richards et al.,[9] results from extensive analysis justify caution in applying this code as designated for common cancer phenotypes; information should only be captured after formal calibration such as dataset-specific personal/family history modeling	–
PM1	located in a mutational hot spot and/or critical and well-established functional domain	no	considered as a component of bioinformatic analysis (PP3/BP4)	–
PM2	Absent/rare from controls in an ethnically matched cohort population sample	yes, supporting weight	only applied for a variant when it is absent from relevant population dataset; extensive analysis to inform weight	Appendix G
PM3	for recessive disorders, detected in trans with a pathogenic variant	yes, variable weight	apply for patient with phenotype consistent with BRCA1- or BRCA2-related Fanconi Anemia (FA) and co-occurrent variants in the same gene; extensive analysis to inform criteria to designate points used to assign final weight	Appendix H; Specifications Table 6

(*Continued on next page*)

Table 1. ***Continued***

Evidence code[a]	Simplified criterion description[a]	Included in BRCA1 and BRCA2 specifications	Extension and other comments	Relevant appendix sections, and specifications figures and tables in ClinGen Specification Registry, with numbering as for original approved specifications[b]
PM4	protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants	no	considered as a component of bioinformatic analysis (PP3/BP4)	–
PM5	missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before	not in original format	this evidence is considered as a component of bioinformatic analysis (PP3/BP4)	
		repurposed use, variable weight	PTC variant in an exon where a different proven pathogenic PTC variant has been seen before; extensive analysis informed exon-specific weights; not applicable to splicing-induced PTCs (i.e., +/− 1,2 variants)	Appendix D; Specifications Table 4
PM6	*de novo* (without confirmation of paternity and maternity) in a patient with the disease and no family history	no	*BRCA1/2*-related cancers occur relatively commonly; no information to calibrate the predictive capacity of *de novo* occurrences	–
PP1	co-segregation with disease in multiple affected family members	yes, variable weight	apply weight as per Bayes score from segregation analysis that captures age-specific penetrance for known pathogenic variants	Appendix I
PP2	missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease	no	high frequency of benign missense variants	–
PP3	multiple lines of computational evidence support a deleterious effect on the gene or gene product	yes	apply for predicted splicing for silent, missense/in-frame (irrespective of location in clinically important functional domain) and for intronic variants outside of donor and acceptor 1,2 sites; SpliceAI ≥ 0.20	Appendix J; Specifications Figure 1A
			for predicted impact on protein function via missense or insertion/deletion—only apply for variants inside a (potentially) clinically important functional domain; assessed using calibrated cutpoints for the BayesDel bioinformatic tool for missense prediction. BRCA1 ≥ 0.28; BRCA2 ≥ 0.30	
PP4	phenotype specific for disease with single genetic etiology	not in original format	–	–
		repurposed use, variable weight	use to capture combined likelihood ratio (LR) toward pathogenicity based on multifactorial likelihood clinical data in the public domain or where there is no appropriate ACMG/AMP code	Appendix B
			clinically calibrated evidence types include co-segregation with disease, co-occurrence with a pathogenic variant in the same gene, reported family history, breast tumor pathology, and case-control data	

(*Continued on next page*)

Table 1. ***Continued***

Evidence code[a]	Simplified criterion description[a]	Included in BRCA1 and BRCA2 specifications	Extension and other comments	Relevant appendix sections, and specifications figures and tables in ClinGen Specification Registry, with numbering as for original approved specifications[b]
PP5	reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation	no	ClinGen recommendation	–
Benign criteria				
BA1	stand-alone allele frequency	yes	specifications around exome vs. genome and read depth informed by LR analysis as well as use of maximum credible population allele frequency (MCAF) calculator; applied for filtering allele frequency $\geq$0.001.	Appendix G
BS1	allele frequency greater than expected for disease	yes, variable weight	specifications around exome vs. genome and read depth; informed by LR analysis as well as use of MCAF calculator; applied at strong for filtering allele frequency $\geq$0.0001, and supporting for filtering allele frequency $\geq$0.00002 and $<$ 0.0001.	Appendix G
BS2	observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age	yes, variable weight	applied in (presumed) absence of features of recessive disease, namely FA phenotype; variable weight determined by age ranges	Appendix H; Specifications Table 8
BS3	Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function.	Yes	assay measures effect via protein-only OR mRNA and protein combined; excludes splicing-only assay data demonstrating no impact on splicing—captured under BP7_Strong (RNA)	Appendix E; Specifications Figures 1C and Table 9
BS4	lack of segregation in affected members of a family	yes, variable weight	apply weight as per Bayes score from segregation analysis that captures age-specific penetrance for known pathogenic variants	Appendix I
BP1	missense variant in a gene for which primarily truncating variants are known to cause disease	not in original format	some missense variants are known to cause disease	–
		repurposed use, strong weight	apply for silent substitution, missense or in-frame insertion, deletion or deletion-insertion variants outside a (potentially) clinically important functional domain; informed by extensive analysis	Appendix J; Specifications Figure 1A
BP2	observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed in cis with a pathogenic variant in any inheritance pattern	no	applied only in the context of BS2	–

(*Continued on next page*)

Table 1. ***Continued***

Evidence code[a]	Simplified criterion description[a]	Included in BRCA1 and BRCA2 specifications	Extension and other comments	Relevant appendix sections, and specifications figures and tables in ClinGen Specification Registry, with numbering as for original approved specifications[b]
BP3	in-frame deletions/insertions in a repetitive region without a known function	no	captured by bioinformatic tool prediction and domain analysis	–
BP4	multiple lines of computational evidence suggest no impact on gene or gene product	yes, using calibrated prediction tools	consider type and location of variant to determine applicability of protein and splicing predictors; only applied to variants inside a (potentially) clinically important functional domain; assessed using calibrated cutpoints for the BayesDel bioinformatic tool for missense prediction and SpliceAI for mRNA splicing prediction; SpliceAI $\leq$0.10 and BayesDel BRCA1 $\leq$0.15; BRCA2 $\leq$0.18	Appendix J; Specifications Figure 1A
BP5	variant found in a case with an alternate molecular basis for disease	not in original format	N/A for co-observation: cases with pathogenic variants in two different known breast-ovarian cancer risk genes have no specific phenotype	–
		repurposed use, variable weight	use to capture combined likelihood ratio (LR) against pathogenicity, based on multifactorial likelihood clinical data in the public domain, or where there is no appropriate ACMG/AMP code	Appendix B and K
		–	clinically calibrated evidence types include co-segregation with disease, co-occurrence with a pathogenic variant in the same gene, reported family history, breast tumor pathology, and case-control data	
BP6	reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation	no	ClinGen recommendation	–
BP7	a synonymous (silent) variant for which splicing prediction algorithms predict neither an impact to the splice consensus sequence nor the creation of a new splice site, and the nucleotide is not highly conserved.	yes	code is applied in addition to BP4 for splicing prediction, to capture the low prior probability of pathogenicity of silent variants	Appendix J; Specifications Figure 1A
		additional use to capture splicing	well-established *in vitro* or *in vivo* functional studies show no damaging effect on protein function as measured by effect on mRNA transcript profile—mRNA assay only. Apply as BP7_Strong (RNA) for intronic, silent, and missense/in-frame variants located outside a (potentially) clinically important functional domain	Appendix E; Specifications Figure 1B

[a]Original ACMG/AMP criteria codes and descriptions are as per Richards et al.[9].
[b]ClinGen Specification Registry for ENIGMA BRCA1 and BRCA2 VCEP: https://cspec.genome.network/cspec/ui/svi/affiliation/50087. Version 1.0 Specifications and Appendix are also reproduced in Data S2.

Table 2. LR toward pathogenicity for BayesDel categories selected for *BRCA1* and *BRCA2* VCEP specifications

Gene	BayesDel score category	Benign[a]	%	Pathogenic[a]	%	LR toward pathogenicity[b]	95% confidence interval	ACMG code weight applicable based on LR	ACMG bioinformatic code and weight recommended for *BRCA1/BRCA2* specifications[c]
BRCA1	≤0.15	946	74%	34	7%	0.10	0.07–0.14	moderate benign	supporting benign (BP4)
	>0.15 and <0.28	183	14%	45	10%	0.69	0.51–0.94	no evidence	no evidence (not met)
	≥0.28	149	12%	377	83%	7.09	6.06–8.30	moderate pathogenic	supporting pathogenic (PP3)
BRCA2	≤0.18	216	74%	21	16%	0.21	0.14–0.33	moderate benign	supporting benign (BP4)
	>0.18 and <0.30	46	16%	25	19%	1.18	0.73–1.90	no evidence	no evidence (not met)
	≥0.30	29	10%	88	66%	6.59	4.40–9.90	moderate pathogenic	supporting pathogenic (PP3)

[a]Benign reference set variants were assumed benign based on no functional impact, and pathogenic reference set variants were assumed pathogenic based on full functional impact. Based on extensive previous calibration analysis, missense prediction is considered relevant only for missense variants located within known (likely) clinically important functional domains, and reference set variants were drawn from these regions only. See Note S1 for more details on selection criteria for the functional reference set and Table S3 for reference dataset used for calibration.
[b]LR calculation is as follows: LR = Pi/Po, where Pi is the proportion of pathogenic variants, and Po is the proportion of benign variants, in a given BayesDel Score Category, calculated separately for each gene. Using *BRCA1* BayesDel score category ≤0.15 as an example: Pi = (pathogenic variants in category)/(total pathogenic variants) = 34/456 = 0.0746; Po = (benign variants in category)/(total variants) = 946/1278 = 0.7402; LR = 0.0746/0.7402 = 0.1008. Note that calculating the LRs from the percentages displayed in the table may not match due to rounding.
[c]Recommendation based on consensus opinion of VCEP members was to conservatively apply bioinformatic evidence at maximum supporting weight.

range category and specifically to compare BayesDel score categories to those recommended for general use by Pejaver et al.,[18] published during the VCEP specification process. Based on analysis of a defined reference set (Table S3), the optimal binary cutpoint for BayesDel score prediction of impact for a missense variant within a clinically important functional domain was 0.27 for *BRCA1* and 0.20 for *BRCA2* (Figure S1). The binary cutpoint values were used to designate the central point for an uncertain zone comprised of <20% of each reference set and for which the outer score categories provided at least moderate evidence toward or against pathogenicity based on estimated LR. The VCEP opted, conservatively, to apply this evidence type at supporting weight only (Table 2). Optimal BayesDel score ranges across three categories for both *BRCA1* and *BRCA2* missense prediction (Table 2) did not align with those recommended for generic use by Pejaver et al.[18] (Table S4). Specifically, use of the Pejaver et al. scale performed very poorly for benign reference set variants, in that a BP4 code (at minimum supporting strength for BayesDel ≤ −0.18) would be assigned to <10% of *BRCA1* and *BRCA2* benign reference set variants, with the large majority having no code applicable. Further, a considerable proportion (29% for *BRCA1*, 36% for *BRCA2*) would be incorrectly assigned PP3 at minimum supporting evidence strength, for BayesDel ≥0.13.

Specification of the frequency codes PM2/BS1/BA1 was informed by a combination of LR-based methods[12] and minimal credible allele frequency estimation[17] as recommended by ClinGen. LR estimation approaches previously used for weighting combined results from functional assays[14] were repeated using an expanded dataset and confirmed applicability of PS3 and BS3 at strong level. Extensive review of the literature and consideration of FA-designated features in GeneReviews (https://www.ncbi.nlm.nih.gov/books/NBK1401/) informed the use of presence or (apparent) absence of FA phenotype for application of codes PM3 and BS2, respectively. Recommendations for use and weighting of segregation data for codes PP1 and BS4 built on methods previously established and enhanced for *BRCA1* and *BRCA2* variant interpretation by the ENIGMA consortium,[8,23] which consider gene-specific, age-specific cumulative risk (penetrance) and background population incidence in assessing variant causality.

The application (i.e., "criterion" description) was completely re-purposed for four codes in consultation with the ClinGen SVI WG after consideration of empirical data on *BRCA1/2*-related clinical features. PM5 was designated to assign exon-specific weights for a premature termination codon (PTC) variant found in an exon in which functional data and/or case-control burden analysis and/or family history burden analysis proves that PTCs in the exon are indeed pathogenic (as justified by VCEP analysis, Figure 2). BP1 was used to capture strong evidence against pathogenicity for a variant outside of a known clinically important functional domain predicted to encode a silent or missense/in-frame substitution only (without known or predicted impact on splicing), with strength assigned from probability based studies,[12] and VCEP consideration of large-scale case-control findings for missense variants.[13,24] PP4 and BP5 were repurposed to capture combined LR estimates toward pathogenicity (PP4) or against pathogenicity (BP5) as derived from calibrated multifactorial likelihood ratio analysis (but excluding any direct statistical measurement of bioinformatic prediction scores to avoid overlap with other computational codes).

Key considerations during development of the *BRCA1* and *BRCA2* specifications

Major points for discussion with key ClinGen SVI WG members before and during documentation of the draft specifications included codes capturing bioinformatic or

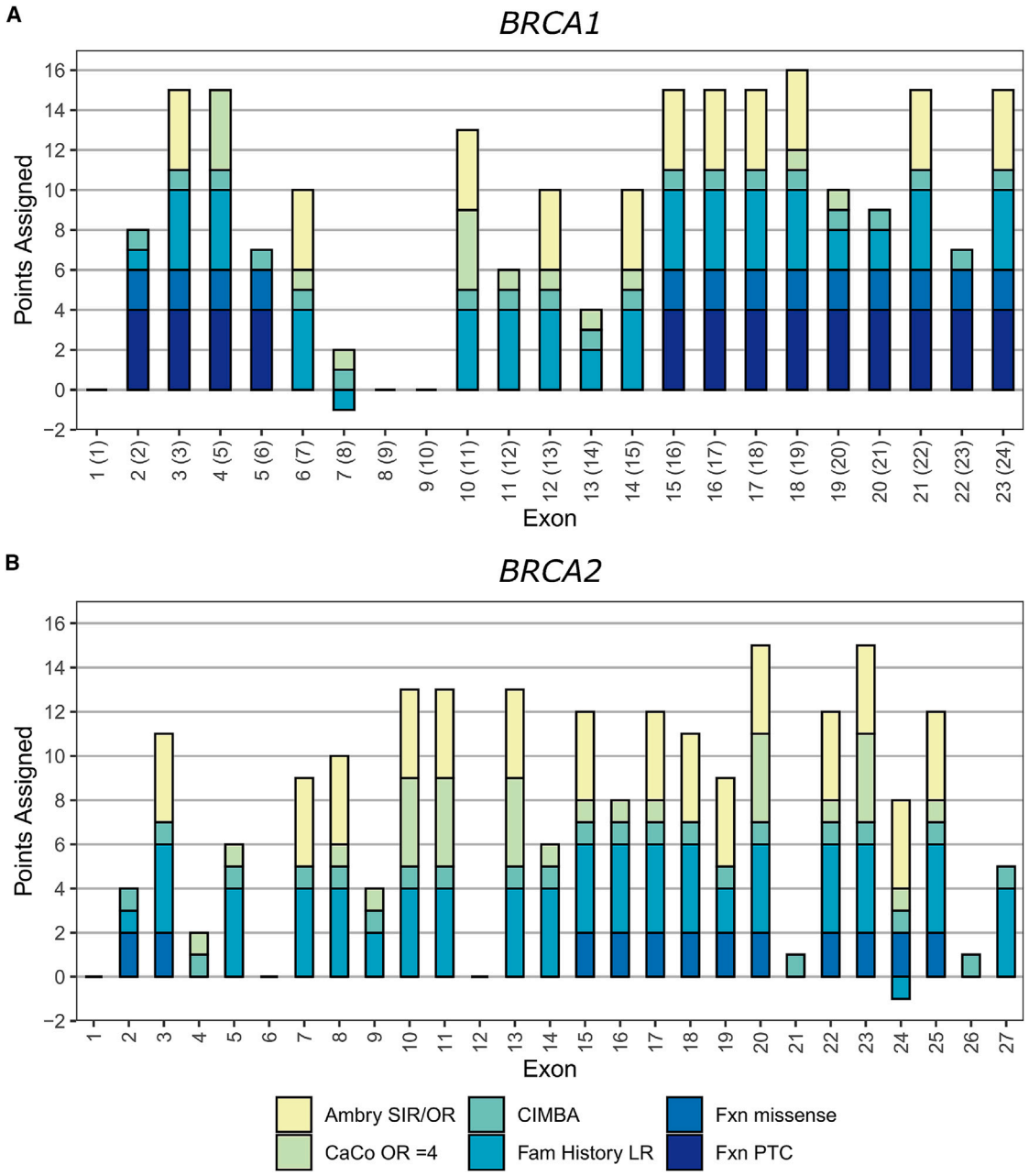

Figure 2. Overview of evidence supporting PM5 exon-specific weights application for premature termination codon variants in *BRCA1* and *BRCA2*

Exon-specific points assigned were derived from per-exon evidence for *BRCA1* (A) and *BRCA2* (B), as detailed in Note S2. Information used to infer evidence and apply points were as follows: observed experimental impact on function for at least one premature termination codon (PTC) variant in an exon (Fxn PTC, assigned 4 points); observed experimental impact on function for at least one missense substitution variant (Fxn Missense, assigned 2 points); case-control odds ratio (OR) ≥ 4.0 estimated for PTC variants observed in a given exon, assigned at full strength for a statistically significant association (4 points), and at supporting strength for non-significant estimates (1 point); family history LR estimates from heterogeneity analysis (fam history LR, assigned points based on LR); standardized incidence ratio (SIR) ≥ 4.0 for PTC variants identified in non-Finnish European (NFE) probands with breast, ovarian, and/or pancreatic cancer compared to gnomAD NFE individuals (Ambry SIR/OR; SIR ≥ 4, $p < 0.05$ assigned 4 points); supporting strength for SIR ≥ 4 non-significant (1 point); and observation of ≥ 5 unique PTCs variant in ≥ 5 families from the CIMBA (https://cimba.ccge.medschl.cam.ac.uk/) highly ascertained cohort of individuals with a *BRCA1* or *BRCA2* pathogenic variant (CIMBA, assigned 1 point). The per-exon evidence was summed across the different evidence types (Fxn PTC, Fxn Missense, CaCo OR ≥ 4, fam history LR, Ambry SIR/OR, CIMBA), to derive an exon-specific evidence strength using a points-based approach (supporting = 1 point, moderate = 2 points, strong = 4 points). Based on the combined evidence, the PM5 (PTC) code can be applied as strong evidence in favor of pathogenicity for most exons. The PM5 (PTC) code can only be applied to germline variants that meet PVS1 codes, namely nonsense and frameshift changes, including large deletions and tandem duplications. Code weight is determined by the exon in which the predicted termination codon occurs. For example, a frameshift variant in *BRCA2* exon 15 that is predicted to result in a PTC within exon 16 would use the code strength of *BRCA2* exon 16 (PM5_Strong [PTC]). Variants in the AG-GT splice site positions, and variants with experimental splicing

(legend continued on next page)

experimental impact on mRNA splicing; these were later resolved in part by review conducted under the umbrella of the ClinGen SVI Splicing Subgroup. Extensive discussion of additional codes, which at the time were new requests for code adaptations by a VCEP, are provided in Table 3.

There was also resolution to align code combinations to achieve variant classes following recommendations arising from Bayesian modeling of the ACMG/AMP classification system[16] to justify expansion of benign code combinations to include benign codes at moderate strength level. To resolve classifications for variants with discordant benign and pathogenic code applications, there was agreement to use the points approach.[25] When using the points approach, the threshold for likely benign was conservatively set at −2 points to align with the original ACMG/AMP specifications that at least two types of evidence are required to reach a classification. In particular, there was concern about assigning likely benign on the basis of bioinformatic evidence alone, which would occur with a threshold of −1 point.

Pilot application of specifications for *BRCA1* and *BRCA2* classification

An overview of the classifications during and after the pilot curation process is shown in Figure 3. Classifications assigned to the 40 pilot variants at the first and second curation phases, and the final classification assigned (with codes applied) are detailed in Table S2. Pre-existing ClinVar classification for the pilot variants, based on all submitter variant assertions at the time of extraction, was as follows: 13 VUSs/conflicting, 11 P, 3 LP/P, 1 LB/B, and 12 B. After initial review, 32/40 variants achieved classification within a confidence band (LP/P or LB/B). Review of the classifications identified several reasons for the differences: new pieces of unpublished internal information used by one biocurator only, unfamiliarity with data presentation, and need for clarification of code use. Between-curator differences often involved recoding of published multifactorial likelihood data to ACMG/AMP codes PP4 and BP5 (23/40), use of frequency information (16/40), use of bioinformatic data (17/40), and weighting of splicing data and use of functional data (15/40). Biocurator feedback indicated the need for more specific advice for some codes, simplified tables and figures in a single "specifications" document, and more detailed recommendations for interpretation of mRNA splicing data. Documentation was revised accordingly, including development and inclusion of an RNA rubric for weighting of mRNA assay data. At this point, additional calibration analysis was undertaken to reassess BayesDel score cut points (as per Table 2), and results were incorporated into the revised specifications.

After re-review in the second curation phase, six of eight variants assigned to "VUS/other" group in the first phase were resolved to a single classification. For variants with classifications that differed in confidence, 5/6 LP/P and all 3 LB/B resolved to a more certain classification (i.e., P or B). Compared to the original ClinVar class, classification was resolved for 11/13 VUS/conflicting variants (5 P, 1 LP, 2 VUSs, 3 LB, 2 B). All variants with pre-existing ClinVar class P (11 variants) or B (12 variants) retained class. Of the remainder, 3 LP/P variants were upgraded to P, and a single LB/B variant was classified as B. As expected, variants annotated with missense or intronic molecular consequences showed greater classification uncertainty and variability (considering conflicts and confidence differences) compared to premature termination codon and synonymous variants in both initial ClinVar classification and at the first VCEP curation step (see Table S2 for details). The complete evidence summary for the final classifications of pilot variants, as submitted to ClinVar, are also shown in Table S2. Detailed examples of how to apply PP1, BS4, PP4, and BP5 based on multifactorial LR analysis, using data as applied for pilot variants, are provided in Table S5.

Further minor revisions of the specifications and appendices were introduced following biocurator feedback and after final review from the ClinGen SVI WG.

Conclusions and future directions

Alignment of pre-existing *BRCA1* and *BRCA2* ENIGMA classification processes with ACMG/AMP classification criteria highlighted several gaps in both the pre-existing processes and in the baseline ACMG/AMP criteria. Statistical calibration of different evidence types was key to justify acceptance—or rejection—of the utility of different ACMG/AMP evidence codes for classification by VCEP members and also the ClinGen SVI WG overseeing VCEP approval. Functional evidence was lacking from the pre-existing ENIGMA external panel criteria for *BRCA1* and *BRCA2* (Data S1) and the requirement to align with ACMG/AMP processes provided motivation for the VCEP to define suitable data sources and reach consensus on specifications for this evidence type. Regarding the codes/criteria deemed not applicable, VCEP member individual opinion concerning the utility of proband-counting criterion was sufficiently contentious that a separate sub study was conducted. This study demonstrated that proband counting with comparison to population datasets is not sufficiently robust for generic application for *BRCA1* and *BRCA2*, given that these genes lead to relatively common diseases.[21] Major items for discussions with key members of the ClinGen SVI WG revolved around the need for ACMG/AMP criteria to be adapted or repurposed to capture more evidence types

data demonstrating introduction of a PTC, do not qualify for PM5 (PTC) code. The points assigned are converted to a code weight using the points system proposed by Tavtigian et al.,[25] which is that 1 point assigned = PM5_Supporting (PTC), 2 points assigned = PM5 (PTC), and 4+ points assigned = PM5_Strong (PTC). Note that the maximum weight applied to PM5 (PTC) is strong, meaning all exons with ≥4 points are assigned PM5_Strong (PTC). The PM5 (PTC) code is considered not applicable for exons with <1 point.

Table 3. Key considerations during development of the BRCA1 and BRCA2 specifications

Key considerations	Additional details
Downgrading PM2 (absence in population databases) to PM2_supporting[12]	calibrated using reference set of variants against gnomAD frequency data to determine appropriate strength for *BRCA1/2*
Adapting the PVS1 decision tree recommendations for weighting predicted loss of function variants[26]	the importance of naturally occurring rescue isoforms[27]
	functional domains designated as clinically important based on location of known pathogenic missense variants
	duplications that preserve reading frame
	splice donor/acceptor ±1,2 dinucleotide variants for exons outside of the coding exons (5′ or 3′ UTRs)[19]
	splice donor +2C>T variants that improve a non-canonical GC site
	splice donor ±1,2 dinucleotide variants that create *de novo* predicted functional "GC" 5′ splice sites[19]
Repurposing PM5 to capture exon-specific evidence as additional information for classification of predicted loss of function stop and frameshift variants	motivated by existing clinical evidence that PTC variants are highly likely to be pathogenic
	collated evidence highlights exons that may be subject to rescue transcripts
	variants in the AG-GT splice site positions causing aberrant PTC transcripts do not qualify for PM5 (PTC) code since the mechanism of impact on mRNA transcripts may introduce variability in proportion of loss of function transcripts produced
Missense bioinformatic predictions should be considered in context of functional domains	missense bioinformatic predictions should be applied only for variants within known clinically important functional protein domains[12]
	location of a missense or synonymous variant (not predicted to impact splicing) outside a known clinically important functional protein domain achieves strong evidence against pathogenicity[12]
	the upweighted repurposed BP1_Strong code for missense, synonymous, and small in-frame variants outside of a known (likely) clinically important protein domain is considered sufficient evidence to achieve likely benign classification for *BRCA1* and *BRCA2* variants
Repurposing PP4 and BP5 to capture likelihood data for multiple evidence types calibrated to predict pathogenicity	expanding potential to provide evidence against pathogenicity for data types previously only considered as positive predictors of pathogenicity, e.g., case-control OR can be applied as PS4 only, but case-control LR estimates could be applied as PP4 or BP5
	increasing the breadth of information types that might be used for variant interpretation, even if not explicitly or directly captured by existing ACMG/AMP criteria, e.g., breast tumor pathology features are not specific to individuals with a pathogenic variant in these genes but are nevertheless predictive of *BRCA1* or *BRCA2* variant pathogenicity (individual pathology data points can provide supporting to moderate evidence, for or against pathogenicity)
	allowing combined likelihoods to be captured under a single code
	facilitating alignment with pre-existing ENIGMA variant classifications based on multifactorial likelihood analysis

(and strengths), in particular to provide evidence against pathogenicity. Agreement by the ClinGen SVI WG to adapt an existing code PM5 provided a mechanism for additional exon-specific weighting so that pre-existing diagnostic laboratory classification practices for *BRCA1* and *BRCA2* PTC variants would not be reversed on introduction of ACMG/AMP classification system (unless indicated by evidence in this process).

The alignment of pre-existing ENIGMA classification methods with ACMG/AMP processes has led to benefits

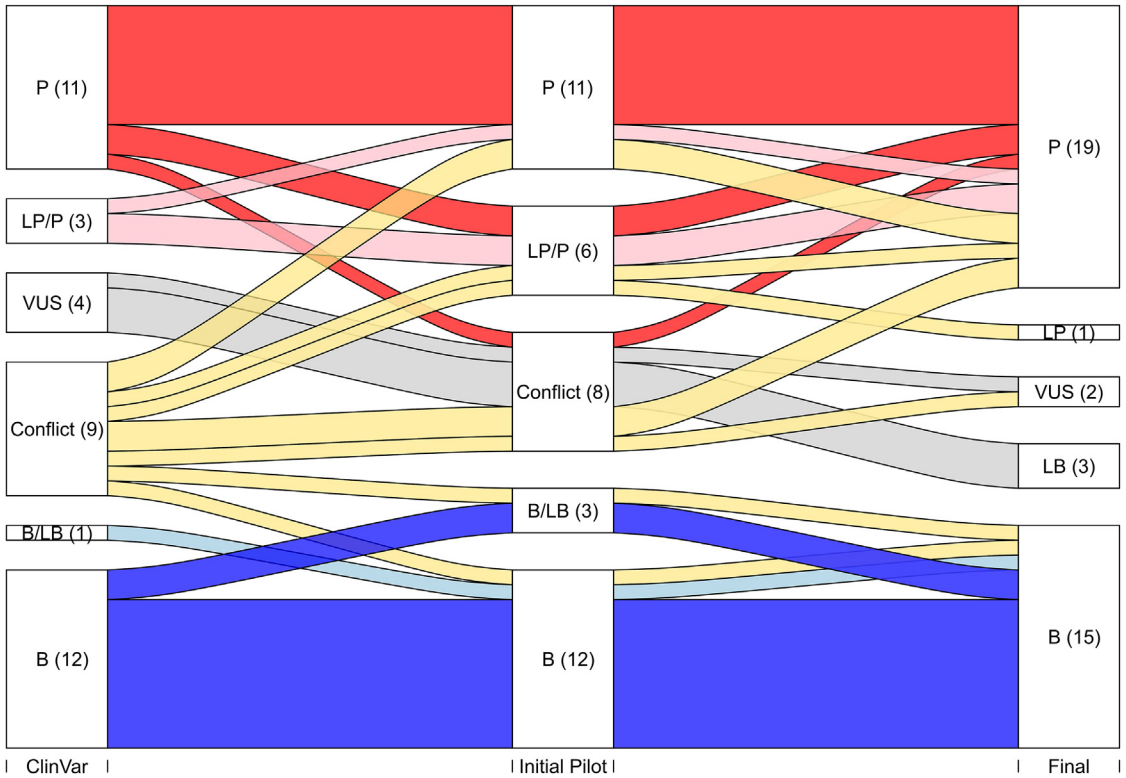

Figure 3. Overview of variant classifications assigned during pilot of VCEP specifications
Sankey diagram shows transition in classification categories for pilot variants from initial ClinVar classification to final classification assigned by the VCEP. The left column represents the initial ClinVar classification category/grouping of pilot variants, namely pathogenic (P), likely pathogenic (LP), variant of uncertain significance (VUS), conflicting (conflict = conflicting), likely benign (LB), and benign (B). The central column represents classification(s) by biocurators after the initial pilot phase. The right column represents the final classifications of pilot variants. For each category/grouping, the number of variants is shown in parentheses. Final VCEP curation resulted in increased certainty in classification for all variants with initial LP/P or B/LB category, movement of three of four variants with initial VUS classification outside of this category, and resolution in classification for the nine variants with initial conflicting classification—eight reaching classification other than VUS.

beyond interpretation of variants in *BRCA1* and *BRCA2*. The research-driven consideration of evidence types and calibration by the ENIGMA *BRCA1* and *BRCA2* VCEP informed the activities of the SVI Splicing Subgroup and has already led to uptake of some of the *BRCA1/BRCA2* specifications for ACMG/AMP criteria by other ClinGen VCEPs. These have included introduction of bioinformatic tiers for splicing prediction, consideration of RNA data under the PVS1 decision process, consideration of read depth for annotation of frequency codes,[28,29] alignment of weights (for recessive disease) with *PALB2*, another gene associated with FA, uptake of a repurposed PM5 code for PTC variants in multiple other genes (including *ATM*, *CDH1*, *PALB2*, and *RUNX1*). It is also notable that some of these adaptations have been taken forward for the draft iteration of the next version of the ClinGen-promoted classification guidelines for application to any Mendelian disease. We also expect to apply similar evidence-based approaches to assess new or updated evidence types for future iterations of the BRCA1 and BRCA2 VCEP specifications, with potential to inform activities of other VCEPs. For instance, VCEP re-appraisal of frequency cut-offs for population frequency-based codes is now warranted given the recent release of a much-expanded dataset encompassed within gnomAD v4. Further, recent calibration analysis of a large-scale breast cancer case sequencing dataset has shown that co-observation of a *BRCA1*, *BRCA2*, or *PALB2* rare variant with a pathogenic variant in another breast cancer panel gene can provide supporting evidence against pathogenicity.[30] Not only can these findings now be put forward for consideration by the relevant VCEPs for future curations, the calibration methods have been made available for application in other contexts.

The ClinGen ENIGMA BRCA1 and BRCA2 VCEP has now initiated an ACMG/AMP-aligned review of *BRCA1* and *BRCA2* variants in ClinVar. As a priority, the VCEP is reviewing variants with conflicting assertions and will shortly reassess pre-existing external expert panel curations to highlight any variants expected to alter in classification after application of the VCEP specifications. VCEP review of all *BRCA1* and *BRCA2* variants in ClinVar will be an extensive and time-consuming effort both due to the enormity of the task (14,665 *BRCA1* and 18,884 *BRCA2* variants as of May 24, 2024) and constraints associated with the rigorous FDA-aligned

ClinGen protocol. For example, after biocurator assessment of information and classification by code assignment and manual entry into the ClinGen Variant Curation Interface,[31] three core approvers are required to review and agree with the classification. The VCEP aims to introduce additional efficiencies to ease the load of biocurators in variant review such as algorithmic code assignment based on frequency and computational information in the BRCA Exchange portal (https://brcaexchange.org/)[32] as a means to prioritize variants for additional data collection and review. This portal will also provide a mechanism for public dissemination of VCEP-aligned ACMG/AMP codes for *BRCA1* and *BRCA2* variants ahead of formal VCEP review.

In summary, this work has provided extensive evidence-based specifications to enable standardized and improved classification of variants in *BRCA1* and *BRCA2*. Further, it has more widely informed improvements in both gene-specific and generic application of the ACMG/AMP classification guidelines.

Data and code availability

The published article includes all datasets generated or analyzed during this study. Unpublished data made available through VCEP members and additional ENIGMA collaborators, including that identified from diagnostic testing laboratories, has been collapsed into de-identified summary form to allow data sharing without compromising patient confidentiality.

Supplemental information

Supplemental information can be found online at https://doi.org/10.1016/j.ajhg.2024.07.013.

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Refer to supplemental information.

Author contributions

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Declaration of interests

M.A. was a paid employee of Invitae. R.C.C. and R.O. are paid employees of Color Health. M.E.R., T.P., and R.K. are paid employees of Ambry Genetics. A.R.M. and M.J.V. received funds from AstraZeneca for contribution to sponsored quality assessments and variant interpretation of *BRCA1* and *BRCA2* VUS (funds paid to the institution).

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