Tracked Version

CanVIG-UK Consensus Specification for Cancer Susceptibility Genes (CSGs) of ACGS Best Practice Guidelines for Variant Classification

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For this tracked version, changes made between v2.18 and v2.19 (current version) are highlighted in red and summarised at the bottom of this document.

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Guidance notes:

- Evidence items for which CanVIG-UK has offered additional specification are shaded in grey. Evidence items are shaded in white where there is no additional specification beyond ACGS Best Practice Guidelines version 4.01 (04/02/2020).
- **Gene specific guidance for specific CSGs can be viewed at *https://www.cangene-canvaruk.org/gene-specific-recommendations* and should be followed for genes where these exist.** These include CanVIG-UK gene specific guidance and gene specific guidance from ClinGen Sequence Variant Interpretation (SVI) Working Groups (+/- notes from CanVIG-UK).
- Evidence items can be combined using evidence (exponent) points for evidence towards pathogenicity (Very Strong= 8, Strong= 4, Moderate= 2, Supporting= 1) or towards benignity (Very Strong= -8, Strong= -4, Moderate= -2, Supporting= -1). Thresholds: ≥10 (Pathogenic), 6-9 (Likely Pathogenic), (-1) – (-5) (Likely Benign), ≤-6 (Benign). It is recommended that evidence criteria and evidence (exponent) scores are included on clinical reports.
- ≥2 concordant evidence items are required for a classification of likely pathogenic/pathogenic/likely benign/benign, with the exception of BA1, which provides standalone evidence towards benignity
- Variants should be reported using HGVS nomenclature, including the clinically appropriate transcript and version number (e.g. MANE select and/or MANE clinical plus) and human reference genome build.
- This specification can be used for single nucleotide variants and insertions/deletions of less than a single gene in size. For insertions and deletions of equal or greater than one gene in size, refer to the ACMG CNV guidance[8].

Tracked Version

Evidence towards Pathogenicity:

Theme: POPULATION DATA		
PS4 (case control): The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. Relative risk or OR, as obtained from case–control studies, is >5.0, and the confidence interval around the estimate of relative risk or OR does not include 1.0.	_VSTR	_MOD
	_STR	_SUP

Vstrong	$P_{exact} \leq 0.0025$
Strong	$P_{exact} \leq 0.05$
Mod	$P_{exact} \leq 0.1$
Sup	$P_{exact} \leq 0.2$

	Cases	Controls	Total
Variant	a	b	a+b
WT	c	d	c+d
Total	a+c	b+d	a+b+c+d

Explanatory Notes:

- Analysis requires non-duplicated, robustly genotyped case data and control data from **equivalent ethnic groups**. If the ancestry of individuals in case and control datasets is known to differ, PS4 cannot be applied at any strength.
- Nationally/regionally collected datasets or published case data may be used but there should be a minimum of 2 case observations for PS4 to be applied (at any strength).
- For Western European case data, comparison to the UK Biobank White population is recommended as it is currently the largest dataset available with comparable ancestry (i.e. 442,266 White individuals from data retrieved January 2023).
- Estimates of UK Biobank denominator count where there is no count for the variant:
    - It is currently recommended that variant frequency is inferred from inspection at a nearby base at which a variant has been called to ensure denominator count approximates estimated size of subject series
    - If there is no nearby base at which a variant has been called, using a denominator of 95% of the population size is recommended (i.e. 95% x 442,266 White individuals = 420,152 individuals) to approximate for the frequency at that base, accounting for failed calls.
    - WES data should not be used for intronic data.
- The P_{exact} is calculated using the Fishers exact 2-way case control comparison
- The P_{exact} does not reflect effect size. Therefore, the Odds Ratio (OR) from the case control comparison (ad/bc) should be consistent with the effect size anticipated for that gene type and the lower 95% confidence interval of the OR should be >1. The OR can be calculated here (tool allows integer or non-integer values).
    - For a 'high penetrance' gene or variant, OR should be >5 for unselected cancer series. For enriched familial cases, a dataset-specific enrichment factor should be used to calculate the OR threshold where available. Otherwise, OR should be >10 for enriched familial cases.
    - For an 'intermediate penetrance' gene or reduced penetrance variant in a high penetrance gene, OR should be >2 for unselected cancer series. For enriched familial cases, a dataset-specific enrichment factor should be used to calculate the OR threshold where available. Otherwise, OR should be >4 for enriched familial cases.
- If the control frequency is 0, the Haldane-Anscombe correction is required to generate an OR (add 0.5 to cells a, b, c, d) (Do not use the Haldane-Anscombe correction for calculation of the P_{exact})

- If there is uncertainty regarding duplicates in the case series, a commensurately more stringent p-value should be applied.
- For non-coding variants, consider use of the WGS partition of UK Biobank (if access available locally). Otherwise, gnomAD v3 may be used.
- Caution should be exercised in using PS4 for CNVs as sequencing approaches/analytical methodologies can result in wide variation in calling of these variant types in NGS/exome/genome data.
    - PS4 can be applied for (i) whole exon or multiexon copy number variants, or (ii) insertions/deletions of 10-50 base pairs. PS4 should not be applied for sub-exonic CNVs of >50bp.
    - PS4 may be applied through case-control analysis as previously described for SNVs, but reduced by one level of evidence strength.

Case-counting

- Where paired numerator-denominator case frequencies are unavailable, a case-counting approach can be applied.
- For extremely specific rare syndromic cancer susceptibility genes, the UK-ACGS rare disease guidance can be applied. Namely: PS4 can be used at a moderate level of evidence if the variant has not been reported in UK Biobank (in a matched ancestral group) and has been previously identified in multiple (two or more) unrelated affected probands/families with a pathognomonic spectrum of disease, or at a supporting level of evidence if previously identified in one affected individual with a pathognomonic spectrum of disease.
    - In most cases, PM2 should be applicable in order to use PS4 for case-counting.
    - For more common or later onset autosomal dominant disorders, variants with very small numbers of cases in UK BioBank (consistent with disease prevalence and severity/age-of-onset) where PM2 cannot be applied and there are multiple reports in the literature of affected patients but insufficient/no case-control data, PS4 application may still be considered at a maximum of supporting.
- Where the phenotype is less specific, a larger number of observations is required before PS4 should be applied when using a case-counting approach. For example, in the CanVIG-UK BRCA1/2 gene guidance for families with a pattern of diagnoses consistent with a hereditary breast and ovarian cancer syndrome, 5 different families are required for PS4_sup and 10 for PS4_moderate.
- Overall, we would recommend that tallying up of specific phenotypic/familial features should generally be incorporated into PP4 rather than PS4, as per CanVIG-UK MMR gene guidance. However, for *TP53, PTEN* and *CDH1*, case-counting of specific phenotypic/familial features under PS4 has been issued via the respective ClinGen expert groups[1-3]
- Where case-counting has been performed, PP4/PM3/PP1 cannot be used if ‘double-counting’ the same specific subphenotype features which rendered the case eligible for use of PS4
- As for case-control analysis, caution should be exercised in using PS4 for CNVs as sequencing approaches/analytical methodologies can result in wide variation in calling of these variant types in NGS/exome/genome data.
    - PS4 can be applied as described for SNV case-counting, however PM2 must also be applied (see using PM2 for CNVs below), and there must also be no overlapping CNVs in the population dataset used for PM2.

- As for case-control analysis, PS4 should not be applied for sub-exonic CNVs of >50bp.

PM2 (rare in controls): Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or ExAC	_MOD _SUP

Use at **Moderate:** where there are 0 observations of the variant across all populations in gnomAD v4.1

Use at **Supporting** where the variant is not absent but is present at a frequency of ≤0.002% (1 in 50,000 individuals, 1 in 100,000 alleles), or CanVIG-UK/VCEP recommended gene-specific frequency, in the relevant portion of gnomAD v4.1 (see explanatory notes below)

Explanatory Notes:

- Note that allele counts from UK Biobank can be retrieved from the CanVar-UK database against the variant searched; for non-SNVs, a spreadsheet of counts is accessible from the CanVar-UK homepage and can be searched manually.
- For PM2_sup in cancer susceptibility genes, we recommend the use of populations of all ancestries from relevant population databases. Where UK Biobank data has already been used for PS4 application, the non-UK Biobank partition of gnomAD v4.1 should be used to calculate variant frequency to avoid "double-counting". Where UK Biobank data has not been used for PS4 application, data from the entirety of gnomAD v4.1 should be used to calculate an overall variant frequency.
- PM2 should not be applied at any level if the variant is observed in >1 individual in any subpopulation dataset of <50,000 individuals (e.g. any non-NFE group in gnomAD v4.1)
- ClinGen Sequence Variant Interpretation (SVI) Working Group recommends applying PM2 criterion at Supporting evidence weighting only. CanVIG-UK (in agreement with ACGS working group) recommends retaining of PM2_Moderate weighting until further ratification of the ACMG guidelines
- Caution should be exercised in using PM2 for CNVs as sequencing approaches/analytical methodologies can result in wide variation in calling of these variant types in NGS/exome/genome data.
  - PM2 may be applied at either moderate or supporting for (i) whole exon or multiexon copy number variants, or (ii) insertions/deletions of 10-50 base pairs.
  - To apply PM2 for CNVs, the variant must be absent (PM2_mod) or below the defined frequency (PM2_sup) in population data from (i) gnomAD v4.1 SNVs **AND** (ii) gnomAD v4.1 CNVs.
  - PM2 should not be applied at any level for sub-exonic CNVs of >50bp, or where PS4 has already been applied using the same population dataset to measure frequency in controls.
  - Where PS4 has been applied for case-control analysis, PM2 may only be applied if the control data used for PS4 has come from a source other than gnomAD v4.1.
- Where base level allele counts for the control dataset are not available as no variant has been observed at that position, allele counts from nearby bases may be used as an estimate, as per recommendations for PS4 above. Caution should be exercised in using PM2 when the number of alleles sequenced with adequate coverage is unknown both for the specific base and for all nearby bases (more likely relevant for non-exonic variants).

- Caution should be exercised in applying PM2 at any level where the patient has ancestry from populations not well represented in the population databases used.

Theme: COMPUTATIONAL AND PREDICTIVE DATA

PVS1 (null variant): Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where LOF is a known mechanism of disease

_VSTR _MOD _STR _SUP

For guidance on application of this criteria see Tayoun et al, 2018 (in particular the PVS1 decision tree and associated notes[4]) and the ACGS Best Practice Guidelines for Variant Classification in Rare Disease 2020 v4 (notes on PVS1 & figure 2)[5].

Explanatory Notes:

- **Start loss variants**: check if a different functional transcript that uses a different start codon exists. If it does, PVS1 may not be applicable at all
- **Stop gain variants within the first 100bp of the first exon:** for these, nonsense mediated decay (NMD) is likely to be evaded and re-initiation of translation may occur using an alternate start codon[6]
  - Identify whether there is another potential in-frame initiation codon downstream; assess the missing N-terminal region of the protein according to the principles described in the decision tree in Tayoun et al 2018[4] to determine the strength of PVS1 (i.e. is the missing region critical to protein function / is it >10% of the entire protein length / are there ≥1 pathogenic variant(s) upstream of the potential initiation codon).
  - If no alternative in-frame start codon is identified, use PVS1 at maximum strength according to the gene-disease relationship.
- **Stop loss variants**:
  - When a frameshift occurs near the end of the gene that abolishes the natural termination codon, and a novel termination codon within the 3'UTR is not predicted; the ribosome may stall at the polyA site and not dissociate. Non-stop mediated decay (NSD) will then be triggered, resulting in a null allele (PVS1_VS)
  - Similarly, NSD and null allele is predicted for base-change variants that abolish the natural termination codon, and where there is no predicted termination codon within the 3'UTR (PVS1_VS)
  - When a frameshift occurs near the end of the gene and a novel termination codon within the 3'UTR is predicted, neither NMD nor NSD is expected to occur and therefore abnormal and extended protein sequence is predicted. In this case, guidance in Tayoun et al 2018[4] should be followed (use PVS1_Strong or PVS1_Moderate depending on functional significance of region and proportion of protein affected)
  - For base change variants that abolish the natural termination codon, and where there is a predicted in-frame termination codon within the 3'UTR: NSD is not predicted and normal protein sequence retained, but extended. In this case, PM4 should be used

- **Variants resulting in a premature termination codon within the last 50bp of the penultimate exon or within the final exon**: these are generally not predicted to undergo nonsense mediated decay.
- **Canonical splice variants at the exon 1/intron 1 donor site and final intron donor/acceptor sites:** should be treated with care. Quantitative RNA studies should be sought to confirm abnormal splice effect.
- **Splicing variants at +2T>C**: may result in functional GC splice sites and PVS1 should be used cautiously in the absence of RNA studies[7]. Use of SpliceAI is recommended to assess the likely impact on splicing.
- **Exon level events such as deletions that are in-frame or not predicted to undergo NMD or duplications not demonstrated in tandem, or ±1, 2 splicing variants where the reading frame is preserved**, are at most a moderate or strong level of evidence, and without published studies may not be eligible for PVS1 at all. Without robust case-control data, these may be difficult to establish as likely pathogenic/pathogenic.
  For variants that require evidence of "region critical to protein function", looking at clinically significant variants in the region can be a good indicator of a functionally significant region. Generally, missense variants demonstrated as pathogenic (and high penetrance) by independent lines of evidence, can be used to upgrade from moderate to strong (assuming they are not acting on splicing). However, care should be taken to determine if variants ascribed as clinically significant have been classified using up to date guidelines. For example, when looking at frameshift or protein truncating variants in databases such as ClinVar, factors such as the date of submission and evidence used should be considered
- **In-frame insertion/deletion events of less than exon size**: refer to PM4 instead of PVS1
- **For single and multi-exon insertions/deletions up to whole gene deletions:** use PVS1 decision tree from Tayoun et al, 2018[4]
- **For large insertion/deletion events involving multiple genes (e.g. detected on microarray or whole genome sequencing):** refer to ACMG copy number variant guidance[8] and SASI guidance for specific cancer susceptibility genes[9]

PS1 (same amino acid change): Same amino acid change as a previously established pathogenic variant, regardless of nucleotide change

_MOD _STR _SUP

Use at **Strong** for a missense or initiation codon variant under evaluation where there is a reference missense or initiation codon variant classified as pathogenic.
Use at **Moderate** for a missense or initiation codon variant under evaluation where there is a reference missense or initiation codon variant classified as likely pathogenic.
Use at **Supporting** for a non-canonical splice variant under evaluation where there is a reference variant at the same base classified as pathogenic/likely pathogenic

Explanatory notes:

- Reference variants must have been classified using ACMG guidance and/or have a 3* classification on ClinVar.
- For variants within the canonical splice site dinucleotide, please refer to PVS1.

- For non-canonical splice variants, the variant under examination should have an equivalent or more deleterious prediction on SpliceAI than the reference variant (equivalent is taken as a difference in scores of ≤0.02 or both reference variant and variant under examination have SpliceAI scores of ≥0.5.
    - For missense variants it is presumed that the REVEL score will be the same as the protein effect is identical).
- PS1 can only be used in conjunction with PS3 (functional data) if the reference variant can be classified as (likely) pathogenic without using functional data
- PS1 cannot be used where the variant under evaluation:
    - has functional data from a BS3_strong/medium-graded assay indicating benignity OR
    - multiple functional assays are contradictory

PM4 (length change): Protein length changes as a result of in-frame deletions/ insertions in a non-repeat region or stop-loss variants — _MOD _SUP

PM4 should be applied with caution in poorly conserved regions. In silico tools such as MutPred-Indel and Ensembl VEP can be used to support the decision to apply PM4.
Use at **Moderate** for

- In-frame insertions/deletions of >1 amino acid
- Stop-loss variants where there is an in-frame termination codon in the 3'UTR and NMD is not predicted

Use at **Supporting** for

- In-frame insertions/deletions of a single amino acid

PM5 (same codon): Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before — _MOD _SUP

Use at **Moderate** if

- Variant under examination has equivalent or MORE deleterious REVEL score than reference variant (equivalent is taken as a difference in scores of ≤0.02) or both reference variant and variant under examination have REVEL scores of ≥0.773[17].

Use at **Supporting** if

- Reference variant is classified as LP AND has only been reported in 1 individual AND/OR
- Variant under examination has a REVEL score of >0.7 and <0.773[17] AND is LESS deleterious than the REVEL score of the reference variant

Explanatory notes:

- Reference variant must have been classified using ACMG guidance and/or have a 3* classification on ClinVar. It should not be predicted to affect function through alterations to splicing
- PM5 can only be used in conjunction with PS3 (functional data) if the reference variant can be classified as (likely) pathogenic without using functional data
- PM5 cannot be used where the variant under evaluation:
    - has functional data from a BS3_strong/medium-graded assay indicating benignity OR
    - multiple functional assays are contradictory

_SUP

PP3 (in silico): Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact)
● Protein impact: ○ Use of a meta-predictor tool such as Revel (>0.7)[10] Use of multiple tools is no longer recommended. ● Splicing impact: ○ Intron-exon boundary: SpliceAI (any Δ score ≥0.2)[11] OR ○ MaxEnt >15% difference **AND** SSFL >5% difference[12]
PM1/PP2 (constraint/enrichment): PP2: Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease. PM1: Located in a mutational hot spot and/or critical and well-established functional domain (e.g. active site of an enzyme) without benign variation _MOD _STR _SUP
● PP2 is applied when encountering a rare missense variant in an individual with the appropriate phenotype where there is enrichment for pathogenic missense variation and constraint for benign missense variation in that **gene** (Z ≥3.09) ● PM1 can be used ***instead*** when the variant lies in a region/domain for which there is greater enrichment for pathogenic missense variation and constraint for benign missense variation ● PP2 and PM1 ***cannot*** be used in combination ● Tools such as Decipher (https://www.deciphergenomics.org/) and Alamut may assist with the identification of functional domains and hot spots containing a high ratio of ClinVar classified pathogenic/likely pathogenic to gnomAD observed variants **Explanatory Notes:** ● Use PP2 at **Supporting** where there is overall constraint for missense variation at the level of the region/exon/gene (Z≥3.09). Where data exists defining regional enrichment, this should be used in place of gene level data (i.e. PM1 in place of PP2) ● Enrichment for pathogenic missense variation and constraint for benign missense variation is best quantified using appropriate likelihood ratios (LRs). Where such data is available, the corresponding evidence level in accordance to the LR should be used. In the absence of LR: ○ Use PM1 at **Moderate** for a variant in a mutational hotspot at which there is no benign variation ○ Use PM1 at **Supporting** for a variant in a mutational hotspot at which there is limited benign variation.

Theme: FUNCTIONAL DATA
PS3 (functional data): Well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product _VSTR _STR _MOD _SUP
This criterion is for ex-vivo variant-specific analyses. Where an assay in the individual patient provides support (e.g. biochemical analysis), this should typically be incorporated within the phenotypic specificity criterion PP4. **Assays of protein function:** ● Variant is considered to have functionally abnormal effect if protein activity assay or functional impact is <25% of wildtype level

- Assay weighting for PS3 should be determined in accordance with Clinical Genome Resource SVI recommendations[13]. Variants used as positive/negative controls should have been classified by an ACMG/expert group as (likely) benign/(likely) pathogenic. See summary of functional studies reviewed by CanVIG-UK in accordance to Brnich et al (2020) principles[13]: an adjusted OddsPath methodology (+0.5 not +1) is recommended in accounting for the incidence of True Positive(s)/Negative(s) and False Positive(s)/Negative(s) in variant validation
- Where data from multiple assays is available:
    - In the instance of conflicts between functional assays of similar evidence strength (STRONG/STRONG, STRONG/MOD, MOD/MOD or Mod/SUP or SUP/SUP) according to evaluation methods described by Brnich et al (2020)[13], refer to the tables below
    - Where concordant, or (as per tables below) there is a permitted discordancy, the evidence level afforded for the combination of the two assays is that of the higher scoring assay
    - Where assays are discordant and of significantly different evidence strengths, the lower-ranked assay should be discarded
    - If differences between functional assay results can be explained by differences in the functional mechanisms incorporated into the assays (for example LOF mediated through an effect on splicing is seen on one assay but the variant appears functional on an assay which would not detect splicing effects), this should not be treated as a conflicting result

Two 'single-element' assays

		Assay 2				
Assay 1		LOF	INT (towards LOF)#	INT (towards FUNC)#	INT (no quantitation provided)	FUNC
	LOF	**PS3**	**PS3**	×	×	×
	INT- (towards LOF)#	**PS3**	×	×	×	×
	INT (towards FUNC) #	×	×	×	×	**BS3**
	INT (no quantitation provided)	×	×	×	×	×
	FUNC	×	×	**BS3**	×	**BS3**

Two assays where Assay 1 comprises multiple sub-elements

		Assay 2			
Assay 1		LOF	INT- (towards LOF)#	INT (towards FUNC)#	FUNC
	All deleterious/ likely deleterious/Intermediate (towards LOF)#*	**PS3**	**PS3**	×	×
	Mixed deleterious/neutral/intermediate	×	×	×	×
	All neutral/likely neutral/Intermediate (towards functional)#**	×	×	**BS3**	**BS3**

#The numeric mid-point of the intermediate range for the functional assay should be used as the cut off for towards LOF vs towards functional
*If no quantitation of intermediate scores is provided, only one intermediate score is allowed. There must be two or more deleterious/likely deleterious results
**If no quantitation of intermediate scores is provided, only one intermediate score is allowed. There must be two or more neutral/likely neutral results

Assays of splicing function:

Evidence Strength	Evidence Points	Assay details
Very strong	8	**2 orthogonal assays:** exhibiting abnormal transcripts; no evidence of leakiness
Strong	4	**1 assay:** exhibiting abnormal transcripts; no evidence of leakiness
Mod	2	**≥1 assay:** exhibiting abnormal transcripts; evidence of some leakiness
Sup	1	**≥1 assay:** exhibiting abnormal/alternative transcripts which have been reported as present in normal controls (implying naturally occurring isoforms)
Do not apply		**≥1 assay:** exhibiting abnormal/alternative transcripts with evidence of extreme leakiness[9]

1. To attain very strong/strong, the criteria by which the disease mechanism is interpreted as loss of function should be met (as per PVS1 recommendations, Tayoun et al (2018)[4])
2. The exon in question must be present in the biologically relevant transcript
3. Assays must be performed in a diagnostically ISO accredited laboratory or recognized research laboratory with which direct consultation can be undertaken. **If evidence is derived from an alternative source (e.g. publication only), downgrade by one level of evidence**
4. All assays should evidence appropriate validations and controls. Laboratory methodology should be appropriately validated: primers must have been tested in ≥5 independent normal control reactions, not necessarily run at the same time (i.e. primers could be validated using 5 normal controls across several runs or runs as a batch on a single run)
5. Experimental data may include quantitative assays (e.g. realtime-PCR, Sanger sequencing with formal quantitation of peak height, tape-station quantification of PCR products, minigene assay, RNAseq using NGS) and semi/non-quantitative assays (e.g. visual evaluation of the relative peak height of Sanger sequencing, gel-based evaluation and visualisation of reverse transcriptase PCR (RT-PCR) products, or analysis for evidence of nonsense mediated decay (e.g. where a SNV in trans with the putative splicing variant appears homozygous on RNA sequencing despite being heterozygous on DNA sequencing, indicating the loss of expression of the transcript containing the putative splicing variant)
6. Combinations of assays deemed orthogonal include (a) two PCR-based assays using different primers (b) ≥2 different platforms e.g. RT-PCR and minigene (c) independent analyses by ≥2 laboratories using the same primers/platform
7. Splicing impact must fulfil one of the criteria below, **otherwise downgrade by one level of evidence**
    a) out of frame + predicted to undergo NMD

b) in-frame but removal of key functional domain or key residues demonstrated by presence of likely pathogenic missense variants in the deleted exon
c) in-frame but removal of >10% of the protein

8. Although there will inevitably be gene by gene and exon by exon variation regarding the lower limit of % normal transcripts ('leakiness') at which normal protein function is maintained, this information is not always known. In the absence of specific data for a given gene/exon, the following thresholds of 'leakiness' should be applied:
   - **No evidence of leakiness**: ratio for allele of >80:20 (abnormal: normal) == overall ratio of >40:60 (abnormal: normal)
   - **Evidence of some leakiness:** ratio for allele of >20:80 (abnormal: normal) == overall ratio of >10:90 (abnormal: normal)
   - **Evidence of extreme leakiness:** ratio for allele of <20:80 (abnormal: normal) == overall ratio of < 10:90 (abnormal: normal). Typically, abnormal transcript will be visible on gel but present only at extremely low level or not visible by Sanger sequencing

   The accuracy of different assays in correctly quantifying ratios of different transcripts will vary and is often poorly quantified. As improved data on the precision of different assays emerges, these standards will likely be amended

   Naturally occurring (i.e. non-pathogenic) splice variants have been catalogued by expert groups for some genes. Please see gene specific recommendations
9. For ±1 or ±2, PVS1 criteria should be used instead of PS3
10. When PS3 is applied for splicing, PP3 (in silico evidence), PM4 (in-frame aberration) and PVS1 (truncating) cannot be applied

Although PP3 cannot be applied alongside PS3, the assay results for variants at the intron-exon boundaries should nevertheless be supported by in silico predictions (MaxEntScan ≥15% difference **OR** SSFL ≥5% difference **OR** SpliceAI (any Δ score ≥0.2)), **otherwise downgrade by one level of evidence.** Exceptions where in silico concordance is not required: (i) U12 splice sites, (ii) TCCTTAAC at the 3' end, (iii) MaxEntScan/SSFL for variants outside of intron-exon boundaries (namely 5': Last 3 bases of exon plus 8 bases on intron 3': 12 bases of intron plus 2 bases of exon)

Theme: SEGREGATION DATA		
PP1 (co-segregation with disease): Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease	_VSTR _STR	_MOD _SUP
See Jarvik and Browning (2016)[14]		

Theme: DE NOVO DATA		
PS2, PM6 (de novo): PS2: De novo (both maternity and paternity confirmed) in a patient with the disease and no family history. PM6: Assumed de novo, but without confirmation of paternity and maternity	_VSTR _STR	_MOD _SUP
See ClinGen SVI Recommendation for de novo Criteria		

Theme: ALLELIC DATA	
	_MOD

PM3 (in trans): For recessive disorders, detected in trans with a pathogenic variant	_STR	_SUP

Use [SVI recommendations for in trans Criterion (PM3)]

Explanatory Notes:

- Comprehensive analysis should be undertaken for the gene to exclude an alternative second pathogenic mutation (e.g. including MLPA) in that gene
- Comprehensive analysis should be undertaken for all other genes for which the phenotypic features overlap
- Requires testing of parents (or offspring) to confirm phase
- Can use for homozygous variants but downgrade by one evidence level
- Caution is required in inferring the pathogenicity for the monoallelic phenotype, as variants may be hypomorphic (e.g. a variant contributing and causing ataxia-telangiectasia may be low penetrance for breast cancer)

Theme: OTHER DATABASES/DATA

PP5 (reputable source): Reputable source recently reports variant as pathogenic

This code is no longer valid. Where required for classification, the specific contributory evidence should be sought directly from the group who has undertaken the variant classification under examination.

PP4 (phenotypic specificity): Patient's phenotype or family history is highly specific for a disease with a single genetic aetiology	_MOD	_STR	_SUP

- PP4 is applied to reflect presence of **clinical or cellular/molecular** 'subphenotypic elements' that strongly implicate the relevant gene (or small gene-set)
- Comprehensive analysis (including CNV analysis) of the gene and related genes should have been undertaken to exclude an alternative pathogenic variant
- Evidence can be summed across multiple families:
  - Total points: Supporting: 1; Moderate: 2; Strong: 4
  - Only one individual per family can contribute
- Where supplied, the inverse evidence must be applied (e.g. if loss of staining for IHC is evidence towards pathogenicity, then retention of staining is evidence against pathogenicity)

LR	Evidence Points	Level	Cellular/molecular phenotype	Example
>**1.4**:1	0.5	-	Moderately predictive for germline aberration of one of a small set of genes	Eg: For *MLH1* variant with MLH1 promoter methylation status unknown • MSI high **AND/OR** • Loss on immunohistochemistry (IHC) of *MLH1+/-PMS2* **AND/OR** Loss of *MLH1* on IHC (*PMS2* IHC status unknown)

>**2.1**:1	1	**Sup**	Highly predictive for germline aberration of one of a small set of genes **OR** Moderately predictive for germline aberration of the specific gene (rare phenotype) **OR** Highly predictive for germline aberration of the specific gene (common phenotype)	Informative LOH at chromosomal locus of tumour-suppressor gene For *MSH2* or *MSH6* variant in colorectal cancer • MSI high **AND/OR** • Loss on IHC of protein pair/appropriate single protein
>**4.3**:1	2	**Mod**	Highly predictive for germline aberration of the specific gene (rare phenotype)	For *SDHB* or *SDHD* variant in phaeochromocytoma/ paraganglioma • Loss of *SDHB* on IHC **AND/OR** • SDH Succinate:Fumarate Ratio high[15]

Explanatory Notes:

For 'clinical' subphenotypic elements

- Use of PP4 is only advised where there has been explicit specification for evidence strength for the relevant 'subphenotypic' element (either via explicit numeric quantitation and/or via explicit guidance)
    - For common, non-specific CSG subphenotypic elements (e.g. aspects of breast and/or ovarian cancer), PP4 should only be used where there has been explicit quantitation for phenotypic specificity (e.g. 'Family History LLR for BRCA1/2, see relevant gene-specific guidance)
    - For rarer CSG subphenotypic elements (e.g. phaeo/PGL), PP4 can be used as per the calculated likelihood ratio for subphenotypic elements (e.g. multiple vs. solitary, familial vs. sporadic, invasive vs. non-invasive)
    - For more specific pleomorphic syndromic CSG presentations for which the clinical subphenotypic elements have been included in the ClinGen Expert Group case-definition for PS4 case-counting (e.g. *CDH1, PTEN, TP53*[1-3]), PP4 cannot be used for clinical subphenotypic elements

For 'cellular/molecular' subphenotypic elements

- Individuals/tumours included must have been demonstrated to carry the germline mutation
- Up to two ***independent*** tumour phenotype assays can be included per case (e.g. MSI AND LOH). Strongly correlated (non-orthogonal) tumour phenotypes from the same case cannot both be included, e.g. MSI and IHC

Evidence towards Benignity:

Theme: POPULATION DATA		
BA1/BS1 (common in controls): Allele frequency is "too high" for disorder (Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium)	_SA	_STR

For use in dominant conditions for alleles of standard penetrance:

Use **BA1** as **Stand_Alone** when the allele frequency in any ethnicity-specific subpopulation of >1000 individuals, or mixed population of >5000 individuals is:

- >1% for well characterised cancer susceptibility genes[16] OR
- The Grpmax Filtering allele frequency (Grpmax FAF) given in gnomAD v4.1 is greater than the BA1 maximum tolerated allele frequency (MTAF) threshold specified for the specific gene by respective expert group (VCEP/CanVIG guidance documents). See explanatory notes below.

Use **BS1** as **Strong** when the Grpmax FAF given in gnomAD v4.1 is greater than the BS1 MTAF threshold (but less than the BA1 MTAF threshold) specified for the specific gene by respective expert group (VCEP/CanVIG guidance documents).

Note that various gene-specific guidance (including *BRCA1/2* and *PALB2*) require only females to be included in the reference population dataset, in this instance the maximum tolerated allele count (AC) should be calculated using the *Calculate AC* tool in cardiodb. Alternatively, the filtering allele frequency (FAF) can be calculated using the *InverseAF* tool in cardiodb which gives a value which can be compared to the MTAF thresholds for BA1/BS1 (see explanatory notes below).

Reference population data from UK Biobank may be used, and the maximum tolerated AC or FAF should be calculated using cardiodb (see explanatory notes below).

Explanatory Notes:

Maximum tolerated allele frequency (MTAF) and filtering allele frequency (FAF) thresholds are used interchangeably in various expert group guidance documents.

Grpmax FAF:

The Grpmax FAF is the lower 95% confidence interval estimate of the allele frequency from the continental subpopulation with the highest FAF (excluding ASJ, FIN, OTH)

The Grpmax FAF is displayed in gnomAD v4.1. The value given is dependent on the dataset chosen, so ensure that the most appropriate population is chosen (e.g. gnomAD v4.1).

There are calculated Grpmax FAF values for both the genomes and exomes dataset in gnomAD; generally the exomes dataset will contain more alleles and this one should be used.

Calculating the maximum tolerated allele count (AC) and filtering allele frequency (FAF):

To determine the maximum tolerated AC, use the *Calculate AC* tool in cardiodb (see training resources from Miranda Durkie for methodology); note in cardiodb, the *Maximum population AF* (MTAF threshold) should be input as a decimal between 0-1 (rather than a percentage). The maximum tolerated AC should be compared to the actual variant allele count in the reference population; if the actual allele count is greater BA1/BS1 can be applied (as appropriate to the input MTAF).

To determine the FAF of a population, use the *InverseAF* tool in cardiodb; the FAF can be compared to the MTAF thresholds for BA1/BS1. If the FAF is greater than the threshold MTAF for BA1 use BA1; if the FAF is greater than the threshold MTAF for BS1 (but less than the BA1 threshold) use BS1. Caution should be applied if using bottlenecked or poorly defined populations in gnomAD (i.e. ASJ, FIN, OTH) as reference populations in the calculation of the FAF / maximum tolerated AC; it is acceptable to use (one or more of) the remaining continental subpopulations as a reference population. Reference populations should be >5000 alleles (mixed) or >1000 alleles (ethnicity-specific).

Theme: COMPUTATIONAL AND PREDICTIVE DATA
BP4 (bioinformatic tools): Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) _SUP
● Protein impact: ○ Use of a metapredictor tool such as Revel (<0.4)[10]. Use of multiple tools is no longer recommended. ● Splicing impact: ○ Intron-exon boundary: SpliceAI (all Δ scores <0.2) OR ○ MaxEnt <5% difference AND SSFL <2% difference AND no evidence of prediction of exonic/deep intronic novel splice site of any strength
BP1: Missense variant in a gene for which primarily truncating variants are known to cause disease _SUP
Use at **Supporting** for genes/gene regions in which >95% of reported pathogenic variants are truncating e.g. APC, PALB2 **Explanatory Note:** Splicing prediction tools e.g. SpliceAI should be applied to exclude potential impact on splicing (see evidence line BP4)
BP7 (synonymous): 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 _SUP
Explanatory Note: BP7 can be applied for the follow variant types, provided (i) they are in regions that are not highly conserved (defined as those with PhastCons score <1 and/or PhyloP score <0.1) and that (ii) BP4 is also met (ie no splicing effect predicted) • synonymous variants • intronic variants at or beyond +7/-21 • non-coding variants in UTRs
BP3 (in-frame deletion): In-frame deletions in a repetitive region without a known function _SUP
Explanatory Note: Particularly relevant to poorly conserved regions. In silico tools such as MutPred-Indel and Ensembl VEP can be used to help support application of BP3.

Theme: FUNCTIONAL DATA			
BS3 (functional data): Well-established in vitro or in vivo functional studies show no damaging effect on protein function or splicing		_STR	_MOD _SUP
• Weighting of BS3 should be determined according to assay criteria defined by Clinical Genome Resource SVI recommendations (Brnich et al, 2020)[13]. Variants used as positive/negative controls should have been classified by an ACMG/expert group as (likely) benign/(likely) pathogenic. See summary of functional studies reviewed by CanVIG-UK in accordance to Brnich et al (2020) principles[13]: an adjusted OddsPath methodology (+0.5 not +1) is recommended in accounting for the incidence of True Positive(s)/Negative(s) and False Positive(s)/Negative(s) in variant validation. **Explanatory Notes:** • BS3 should not be applied for an assay of protein function when in silico tools predict effect on splicing and/or for the first or last three bases of the exon. • A splicing assay can only be used for BS3 for intronic variants and those in the first or last two bases of the exon.			

Theme: SEGREGATION DATA		
BS4 (non-segregation): Non segregation with disease	_STR	_SUP
See Jarvik and Browning (2016)[14] Caution should be exercised in applying BS4 in cancer susceptibility genes associated with common or non-specific phenotypes and where cancers are associated with pathogenic variants in several different cancer susceptibility genes		

Theme: ALLELIC DATA		
BS2/BP2 (observation in trans/cis). BS2: Observation in controls inconsistent with disease penetrance. 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. 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	_STR	_SUP
Use BP2 or BS2 at **Supporting** where no further genotyping or clinical/cellular phenotyping is possible Use BS2 at **Strong** where: • laboratory analysis has been repeated using an orthogonal approach (e.g. different primers) to confirm homozygosity for allele AND • patient is of age at which biallelic variants would be anticipated to be penetrant for a distinctive phenotype AND • patient has been actively examined to exclude relevant phenotype AND/OR had analysis of cellular phenotype OR the homozygote is observed in a specified control population in addition to a heterozygote frequency meeting BS1 Use BP2 at **Strong** where: • alleles have been confirmed as in trans AND • patient is of age at which biallelic mutations would be anticipated to be penetrant for a distinctive phenotype AND		

- patient has been actively examined to exclude relevant phenotype AND/OR had analysis of cellular phenotype

Explanatory Notes:

- BS2 should only be used in the recessive context and for observation of a **homozygote**
- BP2 is used for where the variant is reported as a **compound heterozygote** in conjunction with a pathogenic variant in unaffected individual

For cancer susceptibility genes, **BP2 and BS2** should only be used for those genes in which typical (non-hypomorphic) biallelic variants cause a recognised phenotype that is fully penetrant from infancy. Such genes include *BRCA2, PALB2, MLH1, MSH2, MSH6 and PMS2*

Theme: OTHER DATABASES/DATA

BP6 (reputable source): Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation

This code is no longer valid. Where required for classification, the specific contributory evidence should be sought directly from the group who has undertaken the variant classification under examination.

BP5 (alternative molecular basis): Variant found in a case with an alternate molecular basis for disease _SUP

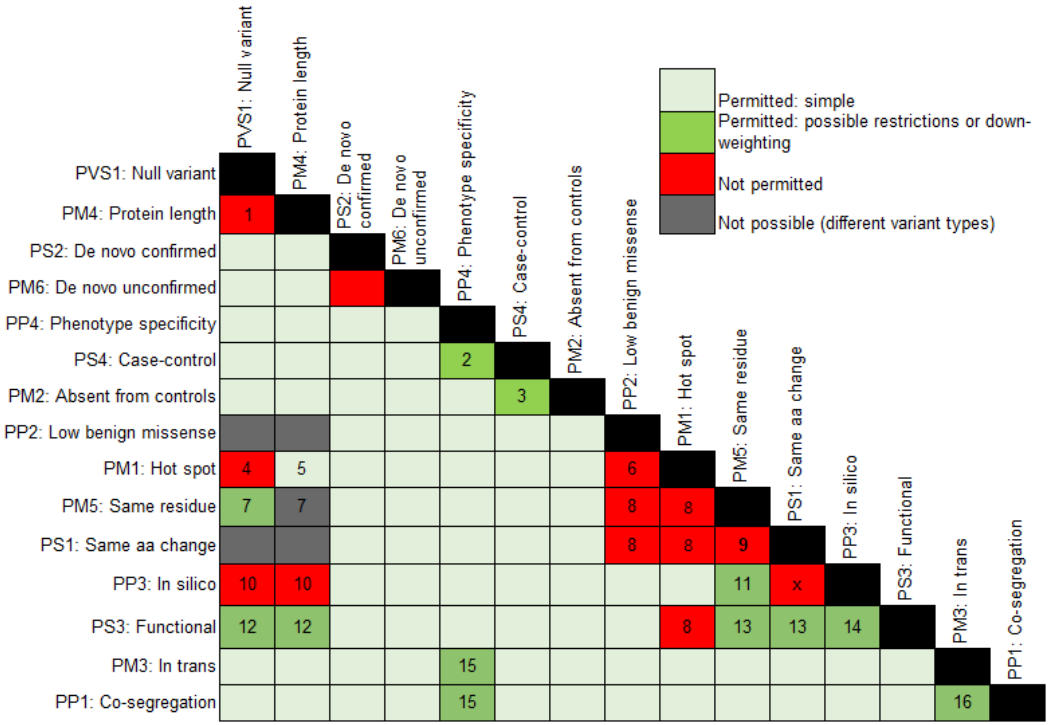
The application of this evidence line is limited in cancer susceptibility genes: only applicable to rare, highly penetrant, dominant syndromic phenotype(s), in which family history is available (e.g. finding of a variant in VHL in a patient with phaeochromocytoma in whom a pathogenic *SDHD* variant is subsequently identified)

Explanatory Note:

This should not be applied for autosomal dominant incompletely penetrant non-syndromic genes associated with common cancers e.g. HBOC (hereditary breast and ovarian cancer). Co-occurrence of ≥2 pathogenic variants in different cancer susceptibility genes is widely reported. Typically, the phenotype exhibited is indistinguishable from that of a single pathogenic mutation.

Tracked Version

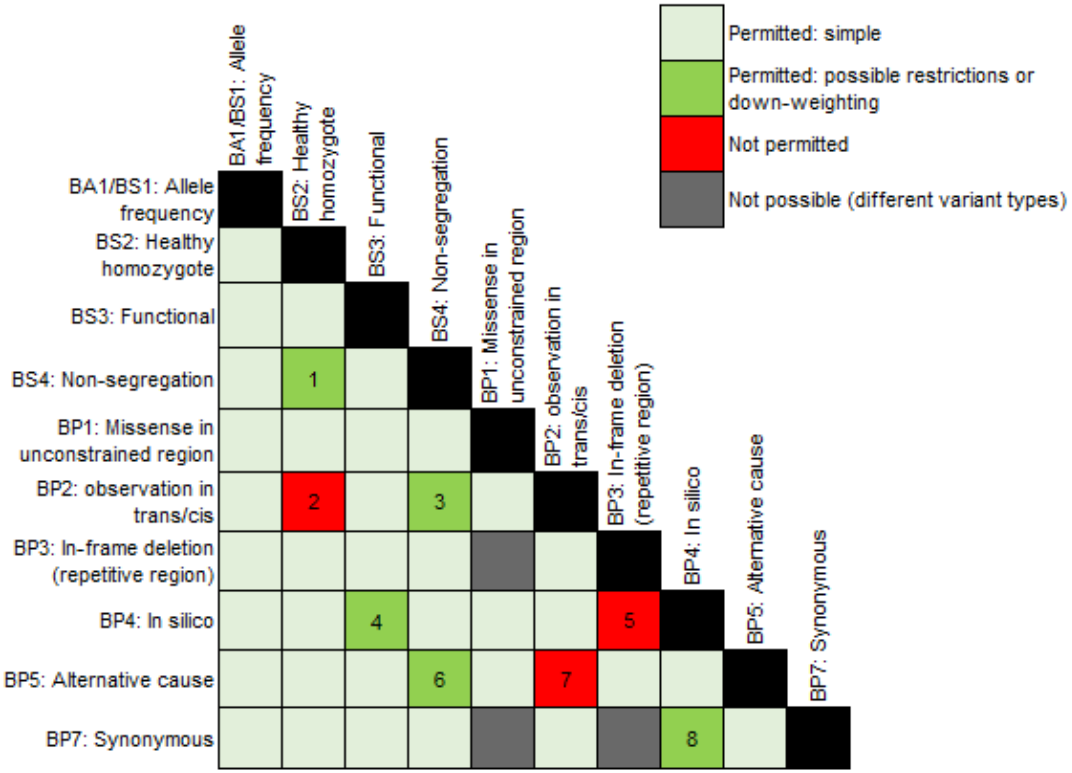
Combinations: towards pathogenicity



1	PM4	PVS1	**Not permitted;** PM4 is used for in-frame deletions/inserions that are small (≤1 exon length), PVS1 is used for larger in-frame multi-exon deletions/duplications
2	PS4	PP4	**Caution:** Can not be combined if case-counting used for PS4 and phenotypic features have been included in case-definition
3	PM2	PS4	**Caution:** The same control series can not be used for PM2 and PS4. Can be combined where a second control series is available
4	PVS1	PM1	**Not permitted;** PM1 is reserved for missense/small insertions-deletions
5	PM4	PM1	**Permitted:** PM1 can be used for missense or small (localised) insertions or deletions
6	PP2	PM1	**Not permitted;** PP2 is used for constraint at gene level, PM1 is used for constraint at domain level. *this has been updated from recommendations in Garett et al 2020 with emergence of more evolved analyses of case-control constraint
7	PVS1	PM5	**Caution;** PM5 application is reserved for missense variants, except where use of PM5 and PVS1 in combination is recommended by gene-specific guidance
	PM4	PM5	
8	PP2	PM5	**Not permitted;** Where points are awarded for substitution at the same residue/ of the same amino acid, additional points can not be awarded for constraint at gene/domain level. A variant for which functional data is used for PS3 or BS3 can not be awarded PM1, as typically functional data informs delineation of a domain/hot-spot.
	PM1	PM5	
	PP2	PS1	
	PM1	PS1	
	PM1	PS3	
9	PS1	PM5	**Not permitted;** PS1 supersedes PM5
10	PVS1	PP3	**Not permitted;** PVS1/PM4 are awarded for pro-pathogenicity mechanism; PP3 can not be used additionally
11	PM5	PP3	**Caution:** PP3 can not be used where PM5 is used at moderate/strong on account of variant having higher Revel score than reference variants
12	PM4	PS3	**Restriction:** Use of a splicing assay within PS3 is not permitted where PVS1 or PM4 are appplied (although a splicing assay may be required for validation of PVS1 or PM4 for an equivocal variant)
	PVS1	PS3	
13	PM5	PS3	**Caution:** PM5 and PS1 can only be applied in combination with PS3 if the reference variant can be classified as (likely) pathogenic without the use of functional data
	PS1	PS3	
14	PP3	PS3	**Restriction:** PP3 can not be awarded for a splicing variant where PS3 has been used (although splice predictions must be consistent with spliceogenic effect)
15	PP4	PM3	**Caution:** PP4 can not be used for subphenotypic elements which themselves have been used in the case-definion of specific phenotype for PM3
	PP4	PP1	**Caution:** PP4 can not be used for subphenotypic elements which themselves have been used in the case-definition of specific phenotype for segregation
16	PM3	PP1	**Caution:** if identification *in trans* is part of evaluation of co-segregation, these elements can not both be counted

Tracked Version

Combinations: towards benignity



1	BS2	BS4	**Caution:** if identification in trans is part of evaluation of co-segregation, these elements can not both be counted
2	BS2	BP2	**Not permitted:** in recessive context BS2 is used for homozygosity; BP2 for heterozygosity
3	BS4	BP2	**Caution:** if identification in trans is part of evidence against co-segregation, these elements can not both be counted
4	BS3	BP4	**Caution:** BP4 can not be awarded for a splicing variant where BS3 has been used (although splice predictions must be consistent with non-spliceogenic effect)
5	BP3	BP4	**Not permitted**: BP3 already recognises sequence context
6	BS4	BP5	**Caution**: BP5 can not be used for alternative explanation where also used in same individual as evidence for non-segregation
7	BP2	BP5	**Not permitted** together
8	BP4	BP7	**Caution:** absence of predicted splicing effect must be confimed using in silico tools

Tracked Version

Revised version	Date	Section	Update	Amended by	Approved by
2.15	***02/12/2021***	PS4	Case counting approach available for BRCA1/BRCA2 genes	Garrett	CStAG
2.15	***02/12/2022***	PVS1	Clarification regarding stop gain variants within the first 100 bp of the gene and use of CNV guidance for large insertions/deletions	Garrett	CStAG
2.15	***02/12/2022***	Combinations	PS3 splicing assays and PM4 not to be used in combination, correction of typo in point 15	Garrett	CStAG
2.15	***04/01/2022***	PS1	Clarification that exact same amino acid change required for strong application	Garrett	Turnbull
2.16	***06/01/2022***	PP4	Amendment of examples for scoring so consistent with MMR gene specific guidance, addition of SDHx example	Turnbull	CStAG
2.17	***28/07/2022***	PS1	PS1 wording change in line with ACGS 2022. Clarification on mechanism of pathogenicity for reference missense variants	Allen	CStAG
2.17	***22/09/2022***	PVS1	Addition of guidance regarding +2T>C variants in PVS1 in line with ACGS	Allen/ Garrett	CStAG
2.17	***22/09/2022***	Guidance notes	Guidance on when to use CanVIG-UK consensus specification and when to use CNV guidance for insertions and deletions. General guidance on use of HGVS nomenclature.	Allen/ Garrett	CStAG
2.17	***22/09/2022***	PS4	Addition of guidance for when ancestry is unknown and minimum number of cases required for PS4 application specified.	Garrett	CStAG
2.17	***22/09/2022***	PS4/PM2	Recommended caution for use of PS4/PM2 for insertions/deletions >10bp. Specified use of DGV Gold and insertion/deletion sizes that are appropriate for PM2_sup.	Garrett/ Allen	CStAG
2.17	***24/11/2022***	PS1	Removal of canonical splice variants and altering strength of application for non-canonical splice variants. Addition of SpliceAI requirement.	Allen	CStAG
2.17	***24/11/2022***	PM5	Specified thresholds for REVEL score difference between reference and variant under examination in line with SVI recommendations.	Garrett/ Allen	CStAG
2.17	***24/11/2022***	PP5/BP6	Removed expert panel application.	Allen	CStAG
2.17	***24/11/2022***	BP7	Specified BP4 must be applied in tandem, and definition added for non-conserved regions.	Allen	CStAG
2.18	***05/05/2023***	PS4	Clarification regarding use of PS4 where PM2 cannot be applied, UK Biobank replaced gnomAD v2.1.1 as recommended population control data source. Rewording of explanatory notes re: case-counting.	Allen/ Garrett	CStAG
2.18	***05/05/2023***	PVS1	Amended link to Tayoun et al. decision tree	Allen	CStAG
2.18	***26/05/2023***	PM2	Updated guidance on population database use and frequency thresholds.	Garrett/ Allen	CStAG
2.18	***03/07/2023***	PM5	Clarification of application of PM5_supporting when REVEL score <0.773.	Garrett	CStAG
2.18	***04/07/2023***	Combinations	PVS1- PM5 combination amended to "caution" due to gene-specific recommendations for protein truncating variants.	Garrett/ Allen	CStAG
2.18	***15/09/2023***	BA1/BS1	Specification regarding use of the gnomAD filtering allele frequency	Callaway/ McDevitt	CStAG
2.18	***28/09/2023***	PVS1	Clarification of PVS1 use for stop codon variants	McDevitt	CStAG

Tracked Version

2.19	***01/05/2024***	PS4	For CNVs: Removed evidence cap at supporting for case-control data, but require downgrade by single level of evidence strength	Allen	CStAG
2.19	***01/05/2024***	PS4	For CNVs: Removed requirement for PS4 case-counting to be downgraded from moderate to supporting, but require PM2 to be applied and require no CNV overlap in population data when applying PM2.	Allen	CStAG
2.19	***01/05/2024***	PS4	Moved CNV case-counting recommendations under the case-counting section of PS4	Allen	CStAG
2.19	***22/02/2024***	PM2	PM2 and PS4 may be used in combination for CNVs if different population-bases control datasets have been used.	Allen	CStAG
2.19	***01/05/2024***	PM2	PM2_moderate may be used for CNVs	Allen	CStAG
2.19	***01/05/2024***	PS4/PM2/ BA1/BS1	Updated population databases and terminology to refer to gnomAD v4.1 (or the UK Biobank partition of gnomAD v4.1 for case-control evidence under PS4)	Allen	CStAG
2.19	***01/05/2024***	PM2	Added note that UK Biobank allele counts for in/dels and SNVs can be found on CanVar-UK	Allen	CStAG
2.19	***23/05/2024***	PM2	Clarification of frequency with respect to both individuals and alleles.	CStAG	CStAG
2.19	***01/05/2024***	PM5	Added clarification that PM5_sup requires REVEL to be >0.7 as well as <0.773	Allen	CStAG
2.19	***01/05/2024***	Combinations	Added clarification that PM1 may not be used with PS3 or BS3	Allen	CStAG
2.19	***01/05/2024***	Combinations	Removed PP5 and BP6 as these evidence codes are no longer valid.	Allen	CStAG
2.19	***13/05/24***	Guidance notes	Requirement of ≥2 concordant evidence items for non-VUS overall classifications	Garrett	CStAG

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