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

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A Garrett[1], L Loong[1], L King[1], M Durkie[2], J. Drummond[3], G.J. Burghel[4], R. Robinson[5], A Callaway[6,7], I. Berry[5], A. Wallace[4], S. Ellard[8], E Baple[8], H. Hanson[1,9], C.Turnbull[1,10]

1) Division of Genetics and Epidemiology, The Institute of Cancer Research, London, UK.
2) Sheffield Diagnostic Genetics Service, Sheffield Children's NHS Foundation Trust
3) East Anglian Medical Genetics Service, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK
4) Manchester Centre for Genomic Medicine and NW Laboratory Genetics Hub, Manchester University Hospitals NHS Foundation Trust, Manchester, UK
5) Yorkshire Regional Genetics Service, Leeds Teaching Hospitals NHS Trust, Leeds, UK
6) Wessex Regional Genetics Laboratory, Salisbury NHS Foundation Trust, Salisbury, UK
7) Human Genetics and Genomic Medicine, Faculty of Medicine, University of Southampton, Southampton, UK
8) Department of Molecular Genetics, Royal Devon & Exeter NHS Foundation Trust, Exeter, UK
9) St George's University Hospitals NHS Foundation Trust, Tooting, London, UK
10) The Royal Marsden NHS Foundation Trust, Fulham Road, London

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 (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.

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:

- The P_{exact} is generated from Fishers exact 2-way case control comparison (ad/bc)
- Analysis requires non-duplicated, robustly genotyped case data and control data from **equivalent ethnic groups**
- For Western European case data, comparison to the gnomAD non-Finnish European (NFE) cancer free population is recommended as it is currently the largest dataset available for the most comparative ethnic group (i.e. 51,377 NFE cancer free individuals for gnomAD v2.1)
- Estimates of gnomAD denominator count:
    - Where there is no count for the variant in gnomAD v2.1.1, it is not possible to ascertain directly from gnomAD the correct frequency for the wildtype allele
    - Estimates of gnomAD denominator count should be inferred from integration of coverage metrics and total number of sequenced samples. Until these metrics are available:
    - 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, we recommend using a denominator of 95% of the population size (i.e. 95% x 51,377 NFE individuals, i.e. 48,808 individuals) to approximate for the frequency at that base, accounting for failed calls. Caution should be taken when applying this approach for intronic variants more than 10bp from exon/intron boundary
- The p-value does not reflect effect size. Therefore, the Odds Ratio (OR) from this 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
    - For a 'high penetrance' gene or variant, OR>5 for unselected cancer series or OR>10 for enriched familial cases
    - For an 'intermediate penetrance' gene or reduced penetrance variant in high penetrance gene, OR>2 for unselected cancer series or OR>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)
- If there is uncertainty regarding duplicates in the case series, a commensurately more stringent p-value should be applied
- For non-coding variants, restriction to the WGS partition of gnomAD is required

Case-counting

- Where paired numerator-denominator frequencies are unavailable, a case-counting approach is required, which takes into account the specificity of phenotype observed in the proband +/- family. For *TP53, PTEN* and *CDH1*, case-counting guidance has been issued via the respective ClinGen expert groups[1-3]; for BRCA1/BRCA2, case-counting guidance has been issued by CanVIG-UK.
- For other rare syndrome 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 gnomAD (in a matched ethnic 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 unrelated affected individual
- 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

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 0 observations of the variant in cancer-free control series >50,000 individuals

Use at **Supporting** where the variant frequency is not absent but at frequency of ≤0.002% in a cancer-free control series of >50,000 individuals (or any equivalent ratio in a larger control series)

Explanatory Notes:

- For cancer susceptibility genes, we recommend the use of cancer-free individuals across populations of all ethnicities in gnomAD v2.2.1 to calculate variant frequency
- PM2 should not be applied at either level if the variant is observed in >1 individual in any subpopulation dataset of <50,000
- 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
- For estimates of gnomAD denominator count where variant is absent, see above as per PS4
- Caution should currently be exercised in using PM2 for non-exonic variants when the number of alleles sequenced with adequate coverage is unknown both for the specific base and for all nearby bases
- Caution should be exercised in using this criterion for small insertions/deletions, as sequencing approaches/analytical methodologies can result in wide variation in calling of these variant types in NGS/exome/genome data
- Caution should be exercised in applying PM2 at moderate 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 _STR	_MOD _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 is likely to be evaded. Assess the function of the remainder of the protein and follow the decision tree in Tayoun et al 2018 (nonsense/frameshift → not predicted to undergo NMD)
- **Stop loss variants**:
    - When a frameshift occurs that does not result in a termination codon in the 3'UTR, the ribosome may stall at the polyA site and will not dissociate; non-stop mediated decay (NSD) will then be triggered (PVS1_VS)
    - For variants creating a protein extension with a termination codon in the 3'UTR (i.e. normal protein sequence is retained but extended), PM4 should be used
    - For frameshift variants with an extension and alteration to coding sequence that are not predicted to undergo NMD or NSD apply then follow guidance in Tayoun et al 2018[4] (PVS1_Strong or PVS1_Moderate depending on functional significance of region and proportion of protein affected)
- **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
- **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 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

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

Use at **Strong** for a missense variant under evaluation where there is a reference variant classified as (likely) pathogenic
Use at **Moderate** for an initiation codon variant under evaluation where there is a reference variant in the initiation codon classified as (likely) pathogenic
Use at **Supporting** for a donor/acceptor splice region variant under evaluation where there is a reference variant at the same base residue classified as (likely) pathogenic
 	
Explanatory notes:
• Reference variants must have been classified using ACMG guidance and/or have a 3* classification on ClinVar. They should not be predicted to affect function through alterations to splicing
• 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
Use at **Moderate** for • In-frame insertions/deletions of >1 amino acid • Stop-loss mutations 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): Novel 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 MORE deleterious REVEL score than reference variant.
Use at **Supporting** if
● Reference variant is classified as LP AND has only been reported in 1 individual AND/OR
● Variant under examination has LESS deleterious REVEL score than 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		

PP3 (in silico): Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact)	_SUP
● Protein impact: ○ Use of meta-predictor Revel (>0.7)[7] Use of multiple tools is no longer recommended ● Splicing impact: ○ Intron-exon boundary: SpliceAI (any Δ score ≥0.2)[8] OR ○ MaxEnt >15% difference **AND** SSFL >5% difference[9]	
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 **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[10]. 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[10]: 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)[10], 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[8]

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)[11]		

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

This is an **exceptional** application, as per UK-ACGS specification. For widely tested cancer susceptibility genes, classifications by large laboratories may have been derived from their substantial series of case data not otherwise publicly available. Laboratories should be contacted directly to procure evidence used in variant classification for evaluation in accordance to ACMG guidelines. Where such evidence **cannot be procured**:

- Any classification of LP/P after 2018 using ACMG classification from
  - ≥2 accredited North American commercial diagnostic laboratories OR
  - ≥1 North American commercial diagnostic laboratory where there is citation of utilisation of otherwise unavailable evidence from their data series OR
  - an approved ClinGen Expert Group (3 star on ClinVar) e.g. INSIGHT, ENIGMA (note cannot be used if evidence from the multifactorial analyses conducted by these expert groups has already been incorporated into other evidence criteria, for example as specified in the CanVIG-UK BRCA1/2 gene specific guidance)

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 MLPA) of the gene and related genes should have been undertaken to exclude an alternative pathogenic mutation
- 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	MSI (for mismatch repair deficiency)
>**2.1**:1	1	**Sup**	Highly predictive for germline aberration of one of a small set of genes	
>**2.1**:1	1	**Sup**		Informative LOH at chromosomal locus of tumour-suppressor gene

			Moderately predictive for germline aberration of the specific gene	Loss on immunohistochemistry of same single protein as variant e.g. MSH6 or PMS2
>**4.3**: 1	2	**Mod**	Highly predictive for germline aberration of the specific gene	Loss on immunohistochemistry of relevant paired mismatch repair proteins e.g. for MSH2 variant loss or loss of MSH2+MSH6
				For MLH1 variant, loss of MLH1+PMS2 on immunohistochemistry **and** normal MLH1 promoter methylation (for MLH1-related mismatch repair deficiency)

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
Use **BA1** as **Stand_Alone** when the condition is dominant and the allele frequency is: • >5% OR • >1% in >1000 western European individuals for well characterised cancer susceptibility genes[12] OR • lower as specified by the respective expert group (BA1 exception list) Use **BS1** as **Strong** when allele frequency in a heterogeneous outbred population is > value specified for specific gene by respective expert group **Explanatory Notes:** ● Where a frequency threshold for BA1/BS1 has been estimated for a given gene/phenotype, the number of observed alleles above which BA1/BS1 can be awarded should be calculated by entering **the observed denominator number of alleles** at the base at cardiodb

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 metapredictor Revel (<0.4)[7]. 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 mutations 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: Splicing prediction tools e.g. SpliceAI should be applied to exclude potential impact on splicing (see evidence line BP4)
BP3 (in-frame deletion): In-frame deletions in a repetitive region without a known function _SUP
Explanatory Note: Particularly relevant to poorly conserved regions

Theme: COMPUTATIONAL AND PREDICTIVE 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 PS3 should be determined according to assay criteria defined by Clinical Genome Resource SVI recommendations (Brnich et al, 2020)[10]. 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[10]: 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)

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 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

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	_STR _SUP

This is an **exceptional** application, as per UK-ACGS specification. For widely tested cancer susceptibility genes, classifications by large laboratories may have been derived from their substantial series of case data not otherwise publicly available. Laboratories should be contacted directly to procure evidence used in variant classification for evaluation in accordance to ACMG guidelines. Where such evidence **cannot be procured**:

- Any classification of LB/B after 2018 using ACMG guidelines from
    - ≥2 accredited North American commercial diagnostic laboratories OR
    - ≥1 North American commercial diagnostic laboratory where there is citation of utilisation of otherwise unavailable relevant evidence from their data series
    - an approved ClinGen Expert Group (3 star on ClinVar), e.g. INSIGHT, ENIGMA (note cannot be used if multifactorial evidence from these groups has been used for other evidence criteria)

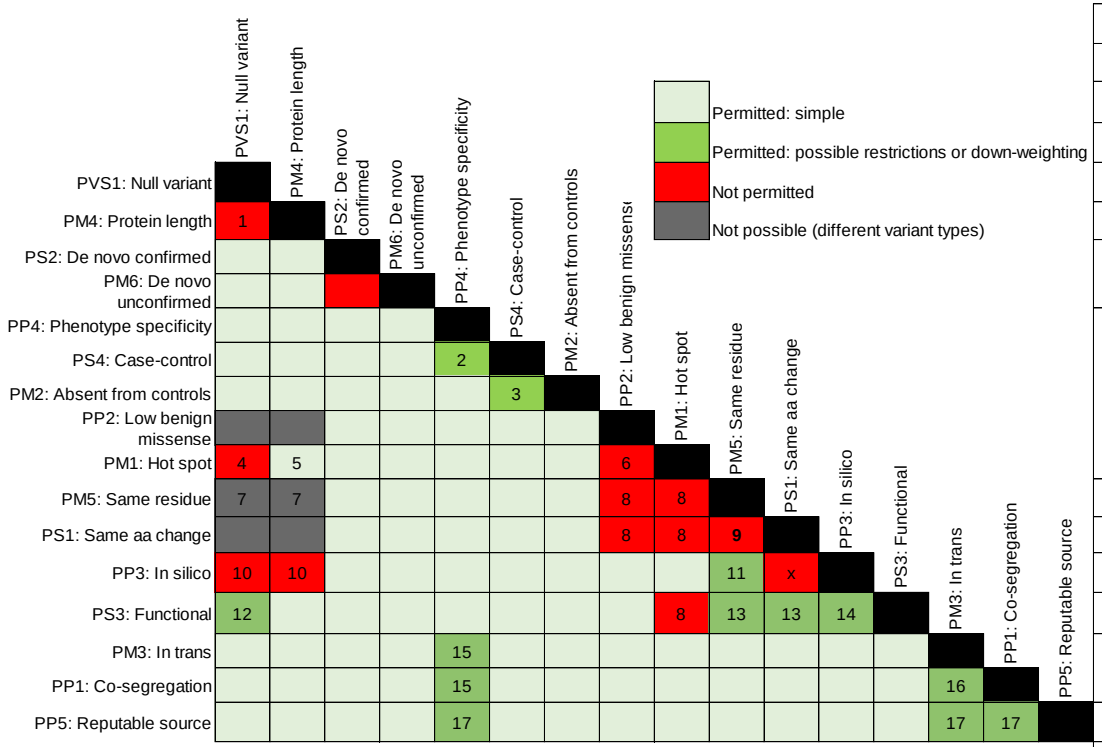
BP5 (alternative molecular basis): Variant found in a case with an alternate molecular basis for disease	_SUP

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:

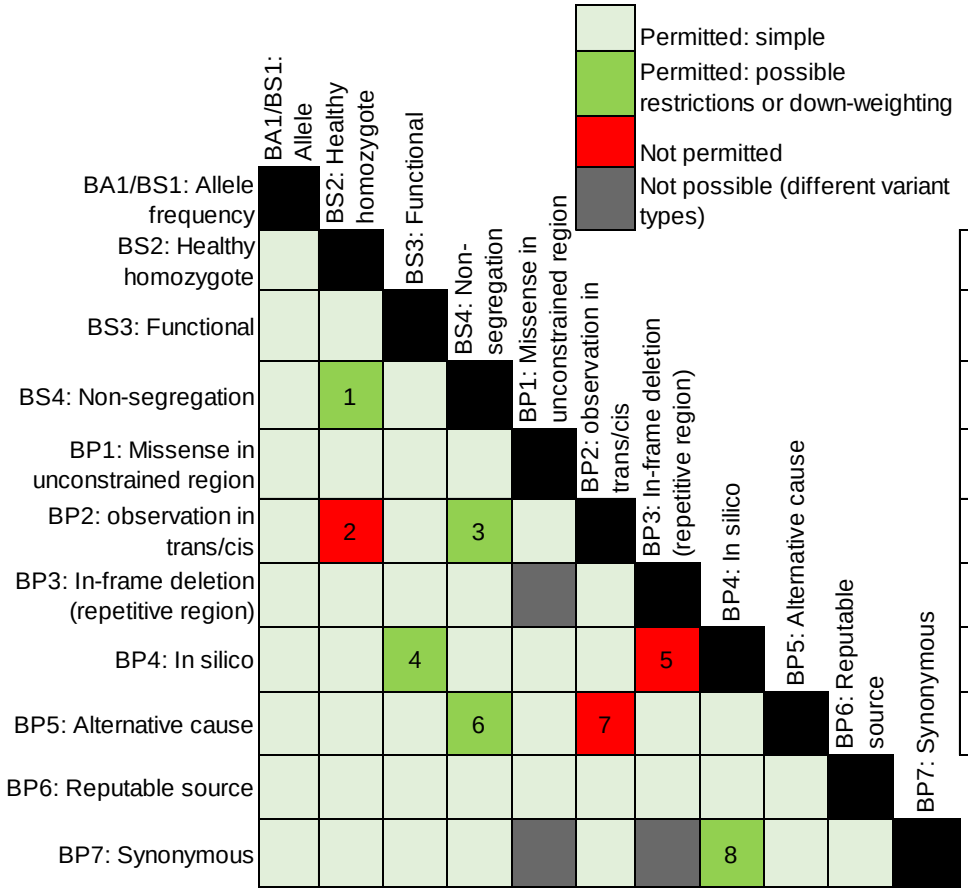
This should not be applied for autosomal dominant incompletely penetrant non-syndromic genes associated with common cancers e.g. HBOC (hereditary breast and ovarian cancer). Co-occurrence of ≥2 pathogenic variants in different cancer susceptibility genes is widely reported. Typically, the phenotype exhibited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

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	**Not permitted;** PM5 is reserved for missense
	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 constaint at gene/domain level. A variant for which functional data is used 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 supercedes 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	PVS1	PS3	**Restriction:** Not permitted for splicing variants (although a splicing assay may be required for validation of PVS1 for an equivocal +1/+2 variant)
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 to 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 to 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
17	PP5	all	**Caution:** PP5 should only be used (i) where the alternative classification is by a large commerical laboratory offering indication of use of 'in-house' data (ii) for multifactorial based classifications (ENIGMA/INSIGHT) where the elements of multifcatorial evidence have not been used elsewhere (PP1, PM3, PP4).

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

References

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