# ACGS Best Practice Guidelines for Variant Classification in Rare Disease 2024

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**Recommendations ratified by ACGS Quality Subcommittee on 20th February 2024**

## 1. Document Version History

| Version | Date | Description |
|---|---|---|
| 2024 v1.2 | 20/02/2024 | Updated guidelines to replace 2020 version - see change log in appendix A |

## 2. Table of contents

## 3. Introduction

Approximately 7,000 rare diseases have been described which in total affect an estimated 1 in 17 of the UK population (approximately 3.5 million individuals). Nearly 5000 of these rare diseases are caused by highly penetrant single nucleotide variants (SNV), small

insertion/deletion (indel) variants (<50bp) or copy number variants (CNV) involving a single gene or region. A genetic diagnosis of a rare disease requires the identification of a disease-causing variant (or biallelic variants in autosomal recessive conditions). A prompt and accurate molecular diagnosis can be crucial to the delivery of optimal care for a patient and their family, including increasingly in targeting treatment (Saunders *et al*., 2012). However, diagnosis of a rare genetic condition can be a challenge and is contingent upon a robust understanding of the molecular aetiology of the disease. The advent of next generation sequencing technology has revolutionised the scale at which genetic testing can be performed, with whole genome sequencing (WGS) increasingly employed as a first line test for patients with rare diseases. Deciphering which, if any, of the observed variants are causative of monogenic disease is challenging as each human genome has 5 million variants (compared to the reference human genome sequence). The new release of gnomAD v4.0 (http://gnomad.broadinstitute.org/) includes SNV data from 807,162 individuals including 730,947 exomes and 76,215 genomes. The dataset also includes approximately 1.2 million high-quality structural variants from genomes (gnomAD SVs v4.0) and in addition, includes 66,903 high-quality, rare (<1%) multi-exon (3 or more exons) autosomal coding CNV sites from 464,297 exomes (gnomAD CNVs v4.0). The continued expansion of gnomAD, ClinVar and other publicly available genomic variant databases, means that maintaining up to date annotation within any analytical pipeline is crucial for informing accurate contemporary variant interpretation, and multiple sources should be used where available. Submission of variants to ClinVar by NHS laboratories in England is now a requirement following completion of the information governance review process.

In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published standards and guidelines for the interpretation of sequence variants (Richards *et al*., 2015). These guidelines describe a framework for classifying variants as “pathogenic”, “likely pathogenic”, “uncertain significance”, “likely benign” or “benign” according to a series of criteria with levels of evidence defined as very strong, strong, moderate or supporting. They recommend that all assertions should be classified with respect to a disease and inheritance pattern. The guidelines also state that a variant of uncertain significance should not be used in clinical decision making. The consequences of a misdiagnosis can be harmful not just for the proband, but also their relatives whose clinical management is altered as a consequence of cascade testing.

In 2016, the ACMG guidelines for germline variant classification and interpretation were adopted by the Association for Clinical Genomic Science (ACGS) for use in UK diagnostic genetic laboratories performing testing for rare disease and familial cancers. The ACGS Best Practice Guidelines for Variant Classification in Rare Disease were developed in 2017 for supplementary use with the ACMG criteria to achieve accurate and consistent use of the guidelines across and within laboratories in the UK and Ireland. These ACGS guidelines include examples to guide practice and notes to provide clarification based on user experience and queries raised by ACGS members. For simplicity, the term SNV guidelines (incorporating all small variants including single nucleotide and small indels) will be used throughout the remainder of this document to refer to the ACMG/AMP guidelines and previous versions of ACGS variant guidelines.

Further development of the ACMG/AMP guidelines has been undertaken through the US ClinGen Sequence Variant Interpretation (SVI) Working Group (Sequence Variant Interpretation

- ClinGen | Clinical Genome Resource). They have released multiple recommendations regarding the application of specific criteria (for example, PVS1 (Abou Tayoun *et al*., 2018), SVI Recommendation for in trans Criterion (PM3) and ClinGen Sequence Variant Interpretation Recommendation for de novo Criteria (PS2/PM6). This group is now working with colleagues from ACMG and AMP to produce a major revision to the Richards *et al*., (2015) SNV guidelines to improve application by the community.

Multiple disease/gene-specific variant curation expert panels (VCEPs) have generated guidelines for their area of expertise. Use of specialist VCEP guidance should be assessed for suitability by the appropriate specialist service and utilised where appropriate for NHS diagnostic practice. Where there are inconsistencies with UK practice, any adaptations required should be agreed with all testing centres, documented and uploaded to the members section of the ACGS website using the available template. For CanVIG assessed and modified VCEP guidelines see https://www.cangene-canvaruk.org/gene-specific-recommendations. Guidelines for non-coding variants based on ACMG criteria have also been published (Ellingford *et al*., 2022).

In 2020, a further set of guidelines specific for the interpretation of copy number variants (CNVs) were updated and released by the ACMG in collaboration with ClinGen (Riggs *et al*., 2020). The guidelines describe a semiquantitative point-based scoring framework for the classification of constitutional germline CNVs and align with the recommendations for sequence variants to classify using the five categories. The guidelines were developed to be applied to any CNV, irrespective of size or technology used for detection. However, it is acknowledged that for some CNVs, the use of the SNV guidelines are more applicable. It is our recommendation that, in the absence of disease/gene-specific VCEPs, the CNV guidelines are adopted for all CNVs, except intragenic deletions and duplications which should follow the SNV guidelines (see **Table 1**). The CNV guidelines have been incorporated into this version of the ACGS guidelines for the first time. We envisage that further development of these guidelines will be available in subsequent versions.

This version of the ACGS guidelines aims to (a) unify the interpretation of SNVs and CNVs in UK laboratories and (b) provide an updated version of the SNV guidelines for use until the major revision of the ACMG/AMP guidelines has been released, tested and is ready for implementation into genomic testing for rare diseases in the UK.

This updated version of the ACGS guidelines also includes guidelines for the reporting of non-coding sequence variants, reduced penetrance, hypomorphic sequence variants and risk alleles.

It is ***essential*** that Clinical Scientists use their professional judgement for variant classification. These guidelines have been developed to augment the ACMG/AMP framework for variant classification. They cannot describe every scenario and the weighting that should be applied for each evidence criterion. Many variants prioritised by a bioinformatic pipeline will not require formal classification. Clinical Scientists must identify those variants that require a full classification, then collate and critically appraise evidence for/against pathogenicity in the context of the individual case under consideration.

**Table 1: Example CNVs and which guidelines to use**

| Copy number variant | CNV or SNV guidelines (critical criteria) |
|---|---|
| **Involving a HI[1] gene or gene where LOF[2] is mechanism** | |
| Deletion with both breakpoints within the gene involving coding exon/s | SNV (PVS1 decision tree) |
| Whole gene deletion including both 5' and 3'UTRs | CNV (2A) |
| Deletion involving the 5'UTR and coding exon/s | CNV (2C-1) |
| Deletion affecting 5'UTR only | CNV (2C-2) |
| Deletion affecting 3'UTR only | CNV (2D-1) |
| Deletion involving the 3'UTR and coding exon/s | CNV (2D-2/3/4) |
| Duplication with both breakpoints within the gene involving coding exon/s | SNV (PVS1 decision tree) |
| Duplication with one breakpoint within the gene | CNV (2J, 2K) |
| **Involving a TS[3] gene** | |
| Whole gene duplication including both 5' and 3'UTRs | CNV (2A) |
| **Multigenic CNV** | |
| Deletion involving multiple contiguous genes | CNV |
| Duplication involving multiple contiguous genes | CNV |

[1]Haploinsufficient gene (loss of one copy of the gene results in a phenotype). [2]Loss of function. [3]Triplosensitive gene (gain of a copy of the gene results in a phenotype).

Please note that these guidelines are primarily intended for general use in classifying highly penetrant variants with Mendelian inheritance patterns including postnatal, prenatal and pregnancy loss setting. Disease-specific guidelines have been developed for disorders where different evidence thresholds are required, for example familial cancer predisposition and inherited cardiac conditions.

## 4. Integration of clinical and scientific data in variant classification

Interpretation of a variant for use in clinical decision making requires comprehensive knowledge of the patient's phenotype, mode of inheritance for the disease gene/region, mutational mechanism (e.g. haploinsufficiency, dominant negative, dosage sensitivity), protein structure/function and the strength of the genotype-phenotype association (Strande *et al*., 2017). Therefore, collaborative working between clinicians and healthcare scientists is key for

high quality variant classification. Clinicians have a responsibility to provide an appropriate level of clinical information to enable clinical scientist interpretation of the genomic variant data.

The prior probability that a patient has a disease-causing variant (or variant pair) in a specific gene/region is important information that is often not available to the laboratory unless the request is for a single gene test or testing is being performed to confirm a suspected clinical diagnosis that is associated with a single gene or small number of genes within a biological pathway or specific syndrome (e.g. Williams syndrome). For disorders where there are clinical diagnostic criteria (for example the Ghent criteria for Marfan syndrome) it is helpful if the referring clinician indicates whether these have been met. When requesting chromosomal microarray testing, large panel tests or exome/genome analysis, it can be very useful for the laboratory if the clinical team provides details regarding the likelihood that a particular clinical presentation is thought to be monogenic, any specific diagnoses that are being considered and where feasible, a shortlist of genes/regions that are thought to be of relevance according to the clinical presentation.

Phenotype specificity is a key evidence criterion for variant interpretation and when testing is undertaken at an exome or genome scale for the diagnosis of very rare disorders, a multi-disciplinary approach is optimal, involving the referring clinician, clinical scientist and other healthcare professionals as appropriate. The purpose of the genomic multidisciplinary team (MDT) meeting is to assess the gene variant(s) identified in the context of the patient's phenotype data and ascertain their contribution to the clinical presentation. The multidisciplinary team (MDT) meeting format is flexible and may be a face-to-face meeting, video or teleconference, telephone conversation or more commonly an e-mail correspondence between a member of the referring clinical team and a laboratory scientist responsible for the case, with other experts included as required.

The key question for the referring clinician/clinical team in an MDT discussion is "Is this patient's clinical phenotype consistent with the genetic variant identified?". If so, what is the strength of the evidence to support the variant classification? For variants of uncertain significance, the clinical team may suggest further tests that result in re-classification of the variant as "likely pathogenic" (or "likely benign"). These might include further genetic or non-genetic tests, clinical investigations and/or co-segregation testing.

There are two categories of evidence within the SNV guidelines that incorporate information regarding the patient (and parental) phenotype; the *de novo* variant assessment, PS2/PM6, and the phenotype specificity, PP4. For CNV guidelines, phenotypic specificity and *de novo* evidence is incorporated into sections 4 and 5.

The *de novo* variant evidence assessment is recorded using the PS2 and PM6 criteria according to the ClinGen SVI recommendation: https://clinicalgenome.org/site/assets/files/3461/svi_proposal_for_de_novo_criteria_v1_1.pdf. PS2 is used when both parental relationships have been confirmed, either through trio exome/genome analysis or using a panel of informative genetic markers. PM6 is used if testing of DNA samples provided by the parents does not identify the variant in either sample but testing for parental relationships by trio exome/genome analysis or using a panel of informative genetic markers has not been undertaken. PS2 and PM6 can only be used if the patient's phenotype is consistent with the disease gene association (see **Table S1, Appendix B** for examples of what

level of evidence should be used). It is also important to consider the possibility that variants in more than one gene are contributing to the patient's clinical presentation (Posey *et al*., 2017). In the CNV guidelines, the scoring for 4A-C and 5A is down-weighted if the variant is assumed *de novo* rather than confirmed.

Of note, in settings where little clinical information is available (e.g. in the context of newborn screening or fetal testing where the phenotypic information is limited) these criteria should not be applied unless or until phenotypic status has been confirmed.

The ACMG/AMP variant classification guidelines may also be applied in interpreting sequence data from patients with common disease phenotypes where the purpose is to identify high penetrance genetic predisposition. Examples include familial breast or colorectal cancer, inherited cardiac conditions and monogenic diabetes. Phenotype and/or family history data are used to estimate the prior probability of a single highly penetrant gene accounting for the majority of the phenotype. Phenotypic information is often used to select patients for genetic testing but additional information to underpin a robust interpretation will often be lacking in the absence of a family history. Caution is needed since (benign) rare variants and common phenotypes may coincide, phenocopies are common and other genetic and environmental factors may influence penetrance. As noted above, different evidence thresholds may be required in these disorders and disease-specific guidelines have been developed for familial cancers (original publication (Garrett *et al*., 2020) and updated versions: https://www.cangene-canvaruk.org/canvig-uk) and inherited cardiac conditions (Kelly *et al*., 2018).

## 5. Variant classification: Supplementary notes for use of the ACMG/AMP sequence evidence criteria and the ACMG/ClinGen copy-number evidence criteria

Variant classification should be undertaken independently from previously published classifications (e.g. those on ClinVar, ClinGen, DECIPHER). Evidence from other laboratories can be requested and the data provided used, alongside data from the current patient, to aid variant classification. Data sources utilised should be saved and stored as an audit trail for any future queries.

The assessment of a variant should include data, including phenotypic details, from all patients identified with the variant to date; the patient referred for testing, previous patients tested in the laboratory, published literature and information from variant databases.

### Small sequence variants

The framework developed by the ACMG utilises a series of criteria in support of a pathogenic (P) or benign (B) evidence. These are described in Tables 3 and 4 in Richards *et al.* (2015). The different types of evidence (functional, variant type, population, *in silico etc*.) are stratified according to the level of evidence (supporting, moderate, strong, very strong) and a pathogenicity classification (pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign or benign).

The ACMG guidelines have been transformed into a quantitative Bayesian framework by Tavtigian *et al*., (2018). Criteria can be easily combined using Bayesian-derived evidence points for pathogenicity (Very Strong= 8, Strong= 4, Moderate= 2, Supporting= 1) or benignity (Strong=

-4, Moderate= -2, Supporting= -1). Thresholds are: ≥10 (Pathogenic), 6-9 (Likely Pathogenic), 0-5 (VUS), -1 to -5 (Likely Benign), ≤-6 (Benign) (Tavtigian *et al.,* 2020). With the exception of BA1 stand-alone, a minimum of two criteria are required to classify a variant as (likely) benign or (likely) pathogenic; therefore, variants with only one piece of evidence e.g. PVS1_vstr (8 points) or BP4_sup (-1 points) are classified as a VUS pending a second corroborating piece of evidence. Use of the Bayesian system allows more flexibility for combining criteria and allows multiple new combinations to be used in addition to those specified in Richards *et al.,* (2015) e.g. 2 str and 1 mod = pathogenic; and therefore in these guidelines, we recommend use of evidence points for combining criteria. It is recommended that evidence criteria and strengths are included in the Appendix of clinical reports, and evidence points may also be added where useful (see example reports on members area of ACGS website https://www.acgs.uk.com/members-area/ and https://www.cangene-canvaruk.org/canvig-uk-report-templates).

The ACMG/AMP guidelines (Richards *et al*., 2015) classify any variant for which there is conflicting evidence, some in support of and some against pathogenicity, as a variant of uncertain significance. This is reasonable when the evidence for and against pathogenicity is of equal strength. Tavtigian *et al.,* (2018) suggest an approach that combines the Bayesian probability but emphasise that expert judgement is always required. For example, it is not appropriate to use *"rare in population studies"* (PM2) and classify a variant as of uncertain significance when all other evidence suggests that it is benign. Garrett *et al.,* (2021) sets out how conflicting evidence can be used during variant classification and gives examples of permissible and non-permissible combinations of codes to avoid double-counting same or similar evidence in cancer susceptibility genes. This has been further developed into a grid for every possible combination of evidence codes in the CanVIG consensus specification available at https://www.cangene-canvaruk.org/canvig-uk-guidance.

**Table 2** describes additional information to assist with the application of the ACMG guidelines. These notes must be used in conjunction with the detailed guidance published by Richards *et al.* (2015) and additional SVI recommendations. The principles of Bayes' theorem apply to variant classification in that each item of evidence in support of or against pathogenicity should be used only once.

**Table 2: Additional information for pathogenic (P) and benign (B) codes usage in SNV guidelines**

| Evidence criteria (level) supplementary notes |
|---|
| **PVS1 – Null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion or duplication) in a gene where LOF is a known mechanism of disease; and non-canonical splice variants where RNA analysis confirms aberrant transcription.**<br><br>The evidence strength level can be modified depending upon the variant type, location within the gene or any additional evidence for the likelihood of a true null effect. A PVS1 decision tree has been developed by the ClinGen Sequence Variant Interpretation group to support the interpretation of loss of function variants (Abou Tayoun *et al*., 2018) and modified for splicing variants by the Splicing SVI (Walker *et al*., 2023) In addition, multiple VCEPs have developed gene-specific PVS1 decision trees.<br><br>Note that caution is required when interpreting 3' nonsense or frameshift variants in the last exon or the last 50bp of the penultimate exon, as these are predicted to escape nonsense mediated decay (NMD). For example the *BRCA2* nonsense variant, p.(Lys3326Ter) c.9976A>T, results in loss of the last 93 amino acids of the BRCA2 protein but does not confer a high or moderate risk of familial breast cancer (Mazoyer *et al*., 1996). |

Nonsense variants within the first 100bp of the first exon are rarely targeted for NMD, and re-initiation of translation may occur using an alternate start codon (Lindeboom *et al*., 2016). If there is a potential in-frame initiation codon downstream, the missing N-terminal region of the protein should be assessed according to the principles described in the decision tree (Abou Tayoun *et al*., 2018) (i.e. is the missing region critical to protein function / is it >10% of the entire protein length / are there any reported pathogenic variants upstream of the potential initiation codon) and apply PVS1 at either reduced strength or n/a, as appropriate. If no alternative in-frame start codon is identified 5' of key domains, use PVS1 at maximum strength.

DECIPHER (https://www.deciphergenomics.org/) protein/genomic display tab shows predicted regions of NMD escape for specific transcripts.

PVS1 can also be used for stop loss variants that abolish the canonical termination codon. In the absence of an in-frame termination codon in the 3' UTR the mRNA transcript is likely to undergo nonstop mediated decay (NSD) and PVS1_vstr can be used. If there is an in-frame termination codon within the 3'UTR then the predicted consequence is a protein with additional amino acids and PM4 (protein length change) can be used (see **Figure 1**).

Frameshift variants in the last exon that cause an alteration to, and extension of coding sequence, and are not predicted to undergo NMD or NSD should follow guidance in Abou Tayoun *et al*., 2018 (PVS1_strong, PVS1_moderate or N/A depending on functional significance of region and proportion of protein affected).

Splicing assays:

New guidelines from the ClinGen SVI splicing subgroup (Walker *et al*., 2023) recommend use of PVS1_*strength* (RNA) for splice variants where RNA studies have confirmed an aberrant splicing profile that can be interpreted using the PVS1 decision tree. PVS1(RNA) is to be used in place of PS3 for splicing assays including RNA analysis from patient material or use of a minigene splicing assay. Considerations for splicing assay design and interpretation of splicing results are included in table S9 of Walker et al., (2023). These include RNA source, use of normal controls, technology etc. Scientific judgement should be used in experimental design and should consider practicalities and the specific clinical question being addressed.

The SVI recommends upgrading PVS1 to vstr for in-frame RNA skipping events encompassing undisputed clinically relevant residues. PVS1 should not be used for variants for which there is a plausible rescue model, based on observation of naturally occurring alternative spliced transcripts e.g. *BRCA1* c.594-2A>C splice acceptor site variant is benign due to an in-frame transcript being a naturally occurring functional isoform (de la Hoya *et al*., 2016).

PVS1 should not be used for non-canonical splice site variants in the absence of RNA studies.

The effect of canonical splice variants at the first and final intron donor/acceptor sites can be difficult to predict. The surrounding sequence should be checked for cryptic splice sites and downgrade evidence if uncertain. See figure 2 of Walker (*et al.,* 2023).

Care should be taken with splice sites predicted to lead to an in-frame transcript or use an alternative cryptic in-frame splice site. Such variants require additional evidence that the region is critical to protein function.

+2T>C splice variants may result in functional GC-AG splice sites and PVS1 should be used cautiously in the absence of RNA studies, particularly in the context of cancer predisposition genes where reduced penetrance and lack of phenotype specificity can complicate variant interpretation (Lin et al., 2020) e.g. *BRCA2* c.8331+2T>C (Nix *et al*., 2022) and *BAP1* c.783+2T>C (Goldberg *et al*., 2021). Use of SpliceAI is recommended to assess the likely impact on splicing; PVS1 should not be applied for +2T>C variants with SpliceAI delta score <0.8 in the absence of RNA evidence.

Approximately 1.5% of natural splice sites use a C at +2 (GC splice site) therefore a +2C>T variant is likely to increase the efficiency of the splice site and PVS1 is n/a - use of SpliceAI is recommended to confirm prediction.

Increasingly, the use of whole genome and long-read sequencing enables the detection and characterisation of complex and structural variants not explicitly covered in the ClinGen SVI guidance (Abou Tayoun *et al*., 2018), such as inversions, translocations, and mobile repeat element insertions. Where such changes impact single genes, we recommend applying PVS1 at appropriate strength based upon the predicted impact on the protein reading frame.

The PVS1 decision tree (Abou Tayoun *et al*., 2018) "assumes that the gene/disease association is at a Moderate, Strong, or Definitive clinical validity level (Strande *et al*., 2017)" in addition to LOF being a known mechanism of disease. Gene-disease validity curations at these levels are available for >1750 genes (https://search.clinicalgenome.org/kb/gene-validity). It is not essential to perform a formal curation for every gene not yet on this list, but laboratories are expected to establish that there is sufficient evidence for the gene/disease association in addition to the LOF mechanism before applying the PVS1 criterion.

For variants not undergoing NMD, functional evidence may be used to uplift the weighting of PVS1 but PS3 should not be used with PVS1_vstrong.

Where gain-of-function (GOF) is the predicted mechanism of disease, PVS1 should not be used. For truncating variants in the last exon of a gene causing GOF effect see PM4.

PVS1 should not be used with PM1, PM4, PP2, PP3.

**PS1 – Same amino acid change as a previously established pathogenic variant regardless of nucleotide change and splicing variants within the same motif with identical predicted effect**

This criterion can be used if there is sufficient evidence for pathogenicity for the same missense variant (i.e. an amino acid change) caused by a **different** base substitution. For example the previously reported variant is p.(Val12Leu) (c.34G>**C**) and your patient's variant is p.(Val12Leu) (c.34G>**T**) as described by Richards *et al.* (2015). PS1_moderate can be applied if the other variant reaches a classification of likely pathogenic. Care should be applied to avoid inappropriate use of this criterion when aberrant splicing is the most likely mechanism of pathogenicity for a putative missense variant. Similarly, this criterion is appropriate to use for nucleotide substitutions in non-coding RNA genes (e.g. *RNU4ATAC*, *SNORD118*) using identical logic.

PS1 may also be used for initiation codon variants where a different nucleotide substitution affecting the initiation codon has been classified as pathogenic and at moderate level where the variant is classified as likely pathogenic.

The ClinGen SVI splicing subgroup (Walker *et al*., 2023) also allows use of PS1 (sup, mod or str) for variants within the same splicing motif or region (donor motif = last 3 bases of the exon and 3–6 nucleotides of intronic sequence adjacent to the exon and acceptor motif = first base of the exon and 3–20 nucleotides upstream from the exon boundary) with same predicted impact. Strength levels also depend on whether the comparison variant is pathogenic or likely pathogenic and the PVS1 weight applicable to the variant under assessment (Walker *et al*., 2023).

PS1 should not be used with PM4.

**PS2/PM6 – *De novo* (both maternity and paternity confirmed) in a patient with the disease and no family history**

This evidence may be provided either from the **patient undergoing testing or a previously identified case**. Note that the genotype **must be consistent** with the phenotype. Mosaicism in either a patient or their parent is evidence of a *de novo* event. If a *de novo* variant was identified by trio exome or genome sequencing, then maternity and paternity will **already** have been confirmed by using a bioinformatics pipeline that would reveal inconsistencies with inheritance. In the situation that a *de novo* variant is identified by trio exome or genome sequencing a cautious approach is recommended (since every exome/genome typically contains between 1-2 *de novo* non-synonymous coding variants and the testing strategy that has been employed will identify these). If the patient's phenotype is non-specific or there is evidence of significant genetic heterogeneity (e.g. intellectual disability), this criterion should only be used at a lower level. See **Table S1, Appendix B** for examples.

A points-based system has been developed by the ClinGen Sequence Variant Interpretation group to enable this criterion to be used at a stronger level for variants that have been shown to have arisen *de novo* in multiple index cases and with differing phenotypic specificity (see https://clinicalgenome.org/site/assets/files/3461/svi_proposal_for_de_novo_criteria_v1_1.pdf.

If PS2/PM6 is applied, the specificity of that patient's phenotype to the relevant disorder, should be captured using an increased strength of PS2/PM6, rather than applying a separate and additional line of evidence within PP4.

**PS3 – Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product**

For use with variant specific protein functional assays only. For gene specific assays e.g. enzyme assay on tissue sample use phenotypic specificity criterion PP4. Protein modelling studies are not considered sufficient evidence for this criterion (but may be incorporated in PM1).

Use of RNA splicing assays has now moved to PVS1(RNA). PS3 should only be applied for well-established assays assessing the impact on protein function not captured by RNA-splicing assays (e.g. *in vitro* or cellular assays) (Walker *et al*., 2023).

Functional studies can include *in vitro* functional assays for specific variants, for example reporter gene assays for transcription factors or saturation genome editing to assay missense variants at scale. ClinGen SVI have produced effective guidelines on application of *in vitro* protein based assays for PS3/BS3 using statistical analysis based on the number of true positive and true negative controls used in the assays and assessment of the quality and confidence of variant-specific assays (Brnich *et al*., 2019). They include a calculator in the "Supplementary

Information" which can be used to calculate the odds of pathogenicity and equivalent strength of PS3. Scientific judgement is still required to assess evidence from other approaches which do not fit this framework (e.g. animal models).

PS3 and PP3 can be applied together for missense variants as the functional assay is assessing protein function/activity and *in silico* tools assess evolutionary conservation, therefore are considered independent.

**PS4 – The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls**

Where large cohort studies and meta-analyses are available, a useful resource for calculating odds ratios and confidence intervals to support the use of PS4_Strong is located at https://www.medcalc.org/calc/odds_ratio.php. gnomAD population data can be used for the control population, although caution may be appropriate when there are many cases of the disorder included in the data set, for example in cardiovascular diseases. Care should also be taken to ensure that case and control populations are ethnically matched where this is possible; note that certain sub-populations are poorly represented in gnomAD.

Case control study data is rarely available for rare diseases, but PS4 can be used as a **moderate** level of evidence if the variant has been *previously identified in multiple (two or more) unrelated affected individuals* or as a **supporting** level of evidence if *previously identified in one unrelated affected individual,* with a **rare and specific phenotype**, **and** has not been reported in gnomAD (see Note 2 in Table 3 (Richards *et al*., 2015)). In practice this is most applicable to autosomal dominant disorders where absence or rarity in the gnomAD database also allows use of both PM2 and PS4. For more common or later onset autosomal dominant disorders, variants with a low frequency in gnomAD, consistent with disease prevalence and severity/age-of onset, should ideally undergo case-control analysis to determine an odds ratio. Where no case-control analysis is possible, and there are a significant number of reports in the literature of affected patients, showing increased prevalence in patients, PS4 can still be cautiously applied in the absence of PM2.

For recessive disorders it is recommended to use PM3 to count rare biallelic cases (if information on their genotype is available), rather than PS4. However, for more common recessive variants, case-control analysis using PS4 may be required to demonstrate enrichment in affected patients versus controls.

**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**

Useful plots of functional domains, gnomAD variants and reported disease-causing variants for a region of a gene are available on the DECIPHER website (see **Figure 2**). *In silico* protein modelling data can be included as supporting evidence.

Evidence of lack of assumed benign missense variation (e.g. depletion of missense variants in the local region in gnomAD) may support PM1 but is insufficient evidence alone for its application (this should generally be applied in the PP2 criterion); at a minimum, one or more of the following should be apparent for PM1 to be applicable:

- Evidence of local enrichment of pathogenic missense variation
- Evidence from protein or protein domain paralogs of pathogenic variation at the paralogous residue
- Evidence that the residue lies in an invariant position in a functionally well-established domain (e.g. enzyme active site)
- Evidence from *in silico* protein modelling studies predicting a likely deleterious structural or ligand-binding impact in a region of the protein with a known function

For regions showing a clear enrichment of pathogenic missense variation but some local benign variation and/or where the functional significance of the protein domain is not well-established, PM1 should not be applied above supporting level.

PM1 may also be applied at supporting level for well-established functional non-coding loci with compelling evidence of a likely impact on gene expression at transcriptional or translational level (Ellingford et al., 2022). This can include variants that impact known functionally or structurally relevant nucleotides in non-coding RNA genes (e.g. *RNU4ATAC*, *SNORD118*). However, the use of PM1 is not appropriate for variants predicted to result in premature termination codons or splicing effects.

PM1 may be upgraded to strong for very specific residues that are critical for protein structure or function. Examples include *FBN1* - affects invariant cysteine in EGF-like calcium-binding domain, *NOTCH3* - Cysteine substitutions that result in an uneven number of cysteine residues within an EGF-like repeat, *COL1A1* or other collagen genes - Glycine substitutions in the triple helix, and cysteine or histidine substitutions in C2H4 zinc fingers such as *GLI3*.

Do not use the same evidence to code PM1 and PM5 or PP2, but the two codes can be used together if each supported by independent evidence.

**PM2 – Absent from controls (or at extremely low frequency if recessive) in Genome Aggregation Database**

In a 2020 recommendation, ClinGen Sequence Variant Interpretation (SVI) Working Group proposed reducing the weight of PM2 to "supporting" by default (SVI Recommendation for Absence/Rarity (PM2) - Version 1.0). The recommendation also indicated that future adjustments to other criteria would be necessary to accommodate the change in PM2 weight. In the ACGS 2020 guidelines, there was continued support for application of PM2 at moderate level, pending a fuller revision of the ACMG guidance. An ACGS mini-impact assessment has been undertaken to assess the effect of downgrading PM2, along with the proposed changes to PP3/BP4 (Pejaver *et al*., 2022) and based on these results (see appendix C) **we recommend no change to PM2_mod use should be implemented, pending the new ACMG guidelines due in 2024.**

For use for autosomal or X-linked dominant disorders where the variant is absent from gnomAD v4.0, or from gnomAD SV v4.0 and gnomAD CNV v4.0, or other high quality WGS dataset, if an intragenic CNV. Scientific judgement may be applied in the situation that the variant is sufficiently rare within gnomAD (rather than absent) for an autosomal dominant disorder where a very low frequency of heterozygotes is consistent with the disease prevalence, penetrance, genetic and allelic heterogeneity. A very useful tool is available at http://cardiodb.org/allelefrequencyapp/ (Whiffin *et al*., 2017) to calculate gene-specific allele frequencies and counts for BA1/BS1. The allele frequency/allele count for PM2 is recommended to be at least a factor of 10 below BS1.

PM2 can be used for autosomal or X-linked recessive disorders if there are no homozygotes/hemizygotes in the relevant gnomAD datasets and the allele frequency is not greater than would be predicted for a benign variant with the disease prevalence, penetrance, genetic and allelic heterogeneity.

Application of PM2 at supporting level may be appropriate where a variant is extremely rare in gnomAD but the published population genetics of the disorder are not sufficiently robust to perform reliable calculations of allele frequency.

Somatic mosaicism of variants in some genes (e.g. *TP53*, *DNMT3A* and *ASXL1*) during hematopoietic clonal expansion (CHIP) can occur with ageing in healthy individuals. The age distribution and variant allele frequency can be checked in gnomAD to help ascertain whether reported variants may be somatic.

PM2 should not be applied:

- If all the other evidence for a variant suggests it is likely benign and application of PM2 would move it into the VUS category.
- For areas of the genome with low coverage.
- For certain variant types (e.g. larger or complex indels, repeat expansion/contractions) which are less readily identified by next generation sequencing.

**PM3 – For recessive disorders, detected *in trans* with a pathogenic variant**

A points-based system has been developed by the ClinGen Sequence Variant Interpretation group SVI Recommendation for in trans Criterion (PM3). PM3 can be used for the current case being assessed (and tallied with other cases) if the patient is compound heterozygous and the other variant is (likely) pathogenic. The system also includes scoring for homozygous variants; we recommend application of the points regardless of the class of the homozygous variant or whether parental testing has taken place to confirm homozygosity (the vast majority of variants will now be identified in the context of NGS assays for which allele drop-out is not a significant risk). Points for homozygous variants are capped at a maximum of 1 point (PM3_mod).

It is acceptable to confirm phase by direct testing of e.g. parental samples, or by using proxy methods (for example, apparent linkage to parentally informative SNPs on NGS reads).

For two rare coding variants observed in gnomAD, it is possible to estimate the likelihood that they are in *cis* (on the same allele), see https://gnomad.broadinstitute.org/variant-cooccurrence. PM3 should not be applied at any level in the context of two variants that predominantly co-occur; unless testing has confirmed they are *in trans*.

For autosomal recessive disorders it is recommended to count rare cases using PM3 rather than PS4. For common recessive variants where PM2 is not applicable, see PS4.

**PM4 – Protein length changes as a result of in-frame deletions/insertions in a non-repeat region or stop-loss variants**

This criterion is used for in-frame deletions or insertions within an exon. Caution is recommended for single amino acid in-frame deletions or insertions where this criterion may be used at a supporting level unless there is gene-specific evidence to warrant use at a moderate level. Selected evolutionary conservation *in silico* predictions tools (e.g. VEST-indel, MutPred-Indel, Ensembl VEP and BayesDel (Cannon *et al*., 2023)) can be useful to assess conservation and confirm whether PM4 is applicable. If the region does not show evolutionary conservation PM4 should not be used.

PM4 should be used for truncating variants in the last exon of a gene causing a gain-of-function effect e.g. *SRCAP, NOTCH2* (Walker et al., 2023).

PVS1 should be used for exon-scale deletions and duplications (including single exon).

Please note that PM4 should not be applied if PVS1 is used.

**PM5 – Missense change at amino acid residue where a different missense change determined to be pathogenic has been seen before**

Where the previously identified missense variant is classified as likely pathogenic and only identified in a single case, we recommend cautious application of this criterion at supporting level only.
The variant being assessed should have a similar or greater predicted impact on the protein than the reference variant. This should be assessed using REVEL score, Grantham distance or BLOSUM62 score.

Where an in-frame deletion or duplication (using the PM4 criterion) overlaps one or more residues in which a known pathogenic or likely pathogenic change has been identified, application of PM5 is reasonable using similar principles. Caution should be applied where the functional consequences of the two variants may reasonably be assumed to be different, and/or where a gain-of-function mechanism may be expected.

Do not use the same evidence to apply PM1 and PM5, but the two codes can be used together if each supported by independent evidence.

**PM6 - Assumed *de novo*, but without confirmation of paternity and maternity**

See ClinGen SVI group points-based table as referenced in PS2.

**PP1 – Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease**

Co-segregation data can be used for autosomal dominant, autosomal recessive, X-linked and imprinted disorders.

The thresholds suggested by Jarvik and Browning (2016) should be used. It is important to consider the number of meioses, **not** the number of informative individuals. Incomplete penetrance, age of onset and phenocopy rates can be incorporated within the calculation. Note that the level of evidence is increased if there are individuals from multiple unrelated families and the number of informative meioses is summed across the families. For example, a supporting level of evidence could be provided either from a single family with three informative meioses or two families each with one informative meiosis or one family with two informative meiosis plus an additional family with one informative meiosis.

In an autosomal recessive disorder where the proband and their sibling is homozygous for a variant and there is a second family where the proband and their sibling are compound heterozygous for the same variant as the first family and a second variant, the segregation information can be combined from both families. However, since co-segregation relates to the allele, the segregation in the second family can only be scored as ½. The information from this scenario would therefore be applied at moderate: ¼ x ½ = 1/8 (>1 family). PP1_supporting can be applied for two families where the proband and their sibling are compound heterozygous and each family is heterozygous for a shared variant: ½ x ½ = ¼ (>1 family). Information from unaffected siblings who are not homozygous for the variant can also be incorporated in the calculation, the probability that an unaffected individual is not homozygous is 1 – ¼ = ¾. Segregation information in autosomal recessive conditions cannot be used where only information about the proband in additional families is known, however this information can be used to apply PM3.

**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**

ExAC constraint scores have previously been used as evidence for a low rate of benign variation (Lek et al., 2016) with Z scores ≥3.09 considered significant. The missense constraint score from gnomAD should now be used (Z

score ≥3.09). However, it is important to consider that there is some evidence for constraint for the region encompassing the variant, not just across the entire gene, PP2 should not be used if there is direct evidence that the local region is not under strong selective constraint.

The DECIPHER database shows regional constraint within the protein view missense constraint track (see **Figure 2**). Havrilla et al., 2018 developed a map of constrained coding regions (CCRs) available as a BED file from https://github.com/quinlan-lab/ccrhtml and online browser tool https://s3.us-east-2.amazonaws.com/ccrs/ccr.ht. The MetaDome web server https://stuart.radboudumc.nl/metadome/ also provides a regional tolerance landscape for proteins. Note that it is not appropriate to use PP2 and consequently classify a variant as being of uncertain significance in the scenario that the allele frequency data within gnomAD would classify as likely benign or benign.

Avoid double-counting evidence for constraint in both PM1 and PP2.

**PP3 – Computational evidence supports a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, *etc*.)**

Evidence-based ClinGen approved threshold scores for missense *in silico* tools have been established and aligned to ACMG evidence strengths ranging from supporting, moderate and strong for PP3 (Pejaver *et al*., 2022). However, based on our impact assessment (appendix C) and decision to not downgrade PM2, **we recommend use of PP3 at supporting strength only** pending further updates to overall ACMG guidelines in 2024 i.e. variants should not be classified as likely pathogenic based on *in silico* (PP3_str) and rarity (PM2_mod) alone.

To predict the impact of missense variants, it is recommended that a single tool is used to avoid introducing bias (Pejaver *et al*., 2022). Meta-predictor tools (e.g. REVEL (Ioannidis et al., 2016) or BayesDel (Feng, 2017)) have been shown to have high positive and negative predictive values and outperform other tools (Tian *et al*., 2019; Cubuk et al., 2021; Pejaver et al., 2022). REVEL scores are commonly used in the UK (ACGS recommended threshold ≥0.7), however do not perform well for all genes e.g. *PKD1*, *DSG2* and genes where the mechanism is gain-of-function (personal communications from ACGS laboratories). A new tool, AlphaMissense, combines structural information from AlphaFold with evolutionary conservation and achieves high auROC compared with other tools (Cheng *et al*., 2023).

PP3 can be used for non-canonical splicing variants. For canonical splice variants see PVS1.

*In silico* splicing prediction tools can be used as evidence to suggest a significant impact on splicing potential for splice site variants. SpliceAI (https://spliceailookup.broadinstitute.org/) using the developer's recommended delta score threshold of >0.2 is likely to be the most accurate single splice prediction tool (Rowlands *et al*., 2021) and (Walker et al., 2023), although alternative tools may perform comparably e.g. MaxEntScan. Note that many commonly used splice prediction tools (such as MaxEntScan, NNsplice and GeneSplicer) have no predictive power for GC-donor splice sites and minor spliceosome (U12) introns; tools such as SpliceAI and SpliceSiteFinderLike are more appropriate alternatives.

Variants affecting the last nucleotide of an exon (if G to non-G) or +5G have an increased prior probability of aberrant splicing (Lord *et al.*, 2019).

PVS1(RNA) should be used if mRNA analysis is undertaken and demonstrates the presence of an abnormal transcript(s) predicted to result in loss of protein expression. In this situation PP3 would not apply as well since the splice prediction is not independent evidence.

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

This evidence criterion incorporates the prior probability that a patient will have a pathogenic variant in a particular gene or genes and therefore does not need to be limited to diseases where there is a single genetic aetiology. This criterion may also be applied in the scenario where a patient has a rare combination of clinical features for which there are a very limited number of known genetic aetiologies and all those genes have been tested. Non-specific phenotypes such as intellectual disability, seizure disorder without a specific EEG pattern and subtle abnormalities of the corpus callosum should never be used in isolation as evidence for PP4. Caution should be exercised when considering phenotypic features which are specific to a disorder that is genetically heterogeneous.

The testing strategy used to identify the variant is also important. For example, when a single gene test has been undertaken because the patient's phenotype is a "good fit" for that specific genetic aetiology, there is a high prior probability that a variant identified within that gene will be causative of the patient's disease and the test specificity is high. In contrast, when a large panel test for a genetically heterogeneous condition is performed, the overall prior probability for finding a causative variant is the sum of the prior probabilities for each individual gene. Using a gene-agnostic whole exome or genome sequencing strategy with variant filtering by mode of inheritance provides significantly increased specificity compared to a gene panel approach and can be cited as additional evidence.

In some situations, it is considered appropriate to use this evidence criterion at a moderate or strong level after MDT discussion (see below and Table S2 appendix B for examples). To use PP4 it is essential that a test has been performed which will identify the majority of known genetic causes of the condition in question.

The specificity of a phenotype may be supported by the presence of a specific constellation of recognisable clinical features consistent with the genetic finding, for example facial gestalt and severe global developmental delay/intellectual disability in a patient with a *NIPBL* variant. Where additional more specific phenotypic features are present this can be used as a moderate piece of evidence (e.g. one of the following additional features; upper-limb reduction defects, growth retardation and microcephaly).

Circumstances where PP4 might be used as a strong piece of evidence include where there is a response to a drug treatment, enzyme activity assay, specific blood indices, methylation signature or muscle biopsy analysis that is pathognomonic of a specific genetic cause of a disorder and would in the absence of genetic confirmation be considered a diagnostic finding.

See CanVIG BRCA1/2 guidelines for incorporation of ENIGMA's multifactorial evidence likelihood ratios using PP4 at variable strengths.

Note care should be taken to avoid double counting the same clinical cases. In recent VCEP guidelines, the use of PP4 has been merged into the PS4 criterion, with cases only counted where they fulfil specific phenotypic criteria for that disorder.

**BA1 – Allele frequency is above 5%**

This can be used as stand-alone evidence of benign impact. For the majority of rare diseases, the Richards *et al.* (2015) threshold of 5% is very high and a more accurate % can be calculated for specific genes using the Cardiodb tool (see BS1) applying disease prevalence, penetrance and genetic heterogeneity, with the allelic heterogeneity fixed at 1.

Used for intragenic CNVs with frequency >1% involving an autosomal dominant disorder with high penetrance. See CNV guidelines for section covering 2F + 2G (Table 1) and 2C-2G (Table 2).

**BS1 – Allele frequency is greater than expected for disorder**

A very useful tool is available to determine whether the allele frequency of the variant is greater than expected for the disorder (Whiffin *et al.*, 2017). In the absence of precise information about the disease prevalence and penetrance we recommend using conservative settings (by selecting the highest likely prevalence and the lowest likely penetrance) to see if the variant frequency on the gnomAD database exceeds the maximum credible allele frequency. The tool can be accessed at http://cardiodb.org/allelefrequencyapp/.

**For an autosomal dominant disorder with high penetrance, it is acceptable to use BS1_Strong as stand-alone evidence to classify a variant as likely benign.**

Caution should be applied for variants in gnomAD v2 with quality flags, use of the latest gnomAD dataset (v4) is recommended for any regions that show poor genotyping quality, low average read depth and/or that reside in gene regions with known pseudogene or paralogs (e.g. *TUBB2B, PRSS1*).

Can be used at strong for intragenic CNVs with frequency >0.5% involving an autosomal dominant disorder with high penetrance; for rare CNVs apply at supporting. See CNV guidelines for section covering 4O (Table 1 & Table 2).

**BS2 – Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age**

Use scientific judgement depending on mode of inheritance, age of onset and disease penetrance.

For example, for a highly penetrant dominant disease BS2 can be used for ≥2 heterozygous healthy (appropriately phenotyped) individuals or ≥2 homozygotes in gnomAD where biallelic phenotype is predicted to be severe and paediatric onset.

In recessive disorders, BS2 can be used for ≥2 appropriately phenotyped healthy homozygotes.
Incidence in gnomAD (phenotype unknown) can also be used for recessive diseases with severe paediatric onset.

For disorders with later age of onset or variable penetrance, a higher number of healthy individuals and/or more detailed phenotyping is required.

BS1 and BS2 can be used together to score a variant as benign.

**BS3 - Well-established in vitro or in vivo functional studies show no damaging effect on protein function**

For protein assays only. For RNA splicing assays see BP7(RNA).

Weighting of BS3 should be determined according to assay criteria defined by Brnich *et al*. (2020).

BS3 should not be applied for an assay of protein function when *in silico* tools predict effect on splicing and/or the variant is located at the first or last three bases of the exon.

**BS4 - Non-segregation with disease**

See Jarvik and Browning (2016). Care should be taken to ensure the phenotype of other affected family members is consistent with proband, and that the disorder does not have common phenocopies e.g. breast cancer, hearing loss etc.

**BP1 – Missense variant in a gene for which primarily truncating variants are known to cause disease**

This criterion is used for missense variants in non-conserved regions of the gene where the mechanism of disease is primarily truncating variants e.g. FAP.

It can also be used for loss of function variants in a gene where the disease is caused by gain of function variants or dominant negative loss of function variants (e.g. those in the last exon of a gene) where there is no evidence of a loss-of-function mechanism and where the gene is not constrained against loss-of-function variation in an appropriate control population (e.g. gnomAD).

It may also be used for missense variants, which do not affect splicing, in genes where missense variants are known to cluster only in key functional domains e.g. *BRCA1* and *BRCA2*.

**BP2 - Observed *in trans* (on different alleles) with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed *in cis* (same allele) with a pathogenic variant in any inheritance pattern**

To be applied for affected cases where a pathogenic variant in a fully penetrant dominant gene that explains the clinical phenotype has been identified. If biallelic variants in that gene cause a different clinical phenotype, the patient must be at an appropriate age at which biallelic pathogenic variants would be anticipated to be penetrant for that phenotype and/or the patient has been clinically assessed to exclude relevant phenotype.

**BP3 - In-frame deletions/insertions in a repetitive region without a known function**

Assess nucleotide and protein conservation, function of region and variant frequency in population controls. Use of *in silico* tools MutPredIndel, VEST etc may also help with application.

**BP4 – Multiple lines of computational evidence suggest no impact on gene or gene product**

We recommend use of BP4 for missense variants with REVEL score <0.4.
For splicing variants, Splice AI delta score <0.1 OR MaxEnt <5% and SSFL <15%.
This criterion should not be used when there is evidence that *in silico* tools do not show satisfactory performance for prediction of pathogenic variants in that gene.

BP4 should not be applied for splicing variants where RNA evidence has already been used in BP7(RNA).

**BP5 - Variant found in a case with an alternate molecular basis for disease**

Caution should be exercised for genes in which co-occurrence of pathogenic variants is reported with no/little impact on clinical phenotype e.g. *BRCA1* and *BRCA2* pathogenic variants can co-occur and do not show any significant impact on cancer type or age of onset.

Caution should also be exercised if the PV identified does not explain all the clinical features or if the patient has a blended phenotype incorporating elements of both genetic diagnoses.

**BP7 – A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved; splice variants where RNA studies have confirmed no impact**

BP7 can also be used for intronic variants at or beyond +7/-21 (where BP4 is also applicable), and non-coding variants in UTRs.

Synonymous variants with no predicted effect on splicing and the nucleotide is not highly conserved can be classified as likely benign using BP4 and BP7.

Not highly conserved regions are those with e.g. PhyloP score <0.1.

ClinGen Splicing SVI group recommend use of BP7 (RNA) for intronic variants and synonymous variants shown to have no effect on splicing by RNA studies. When a substitution is confirmed to have no impact on splicing by a suitable *in vitro* assay, BP7 can be upweighted to BP7_Strong (RNA).

## Figure 1: Use of PVS1 and PM4 for start-loss, stop-loss and truncating/frameshift variants in the last exon

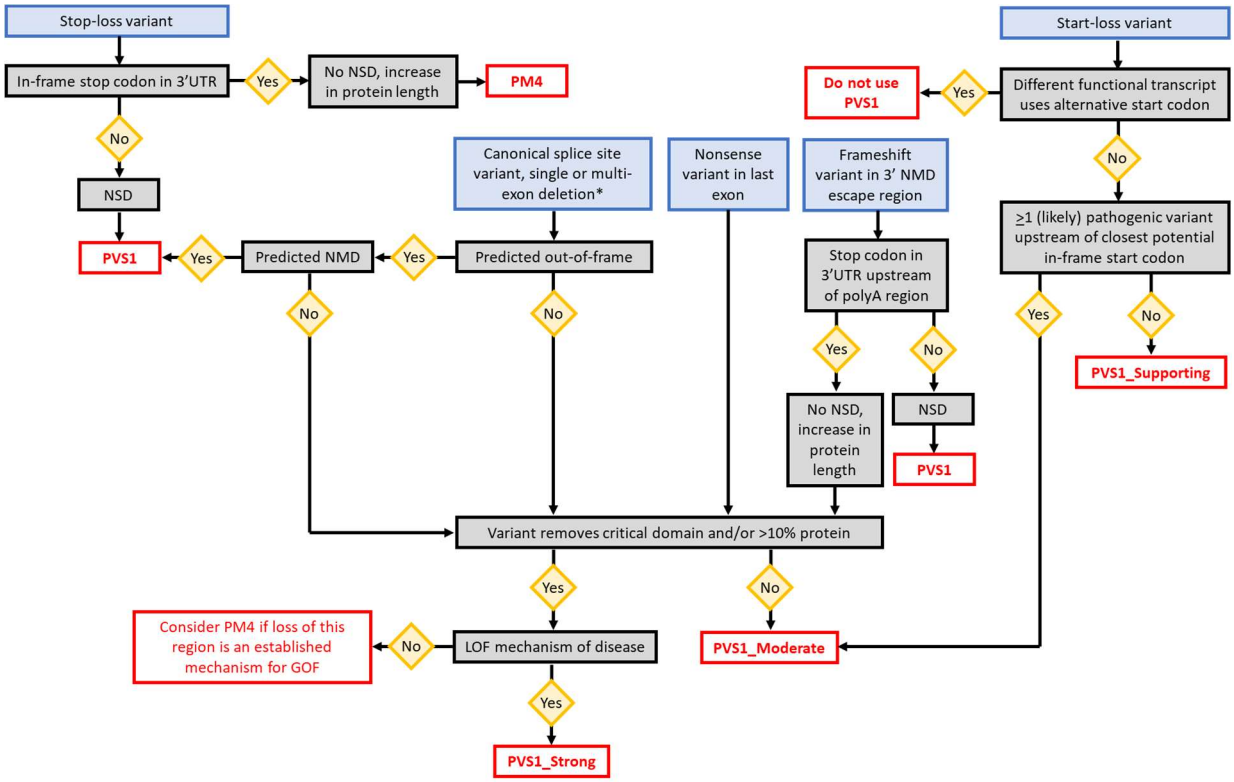
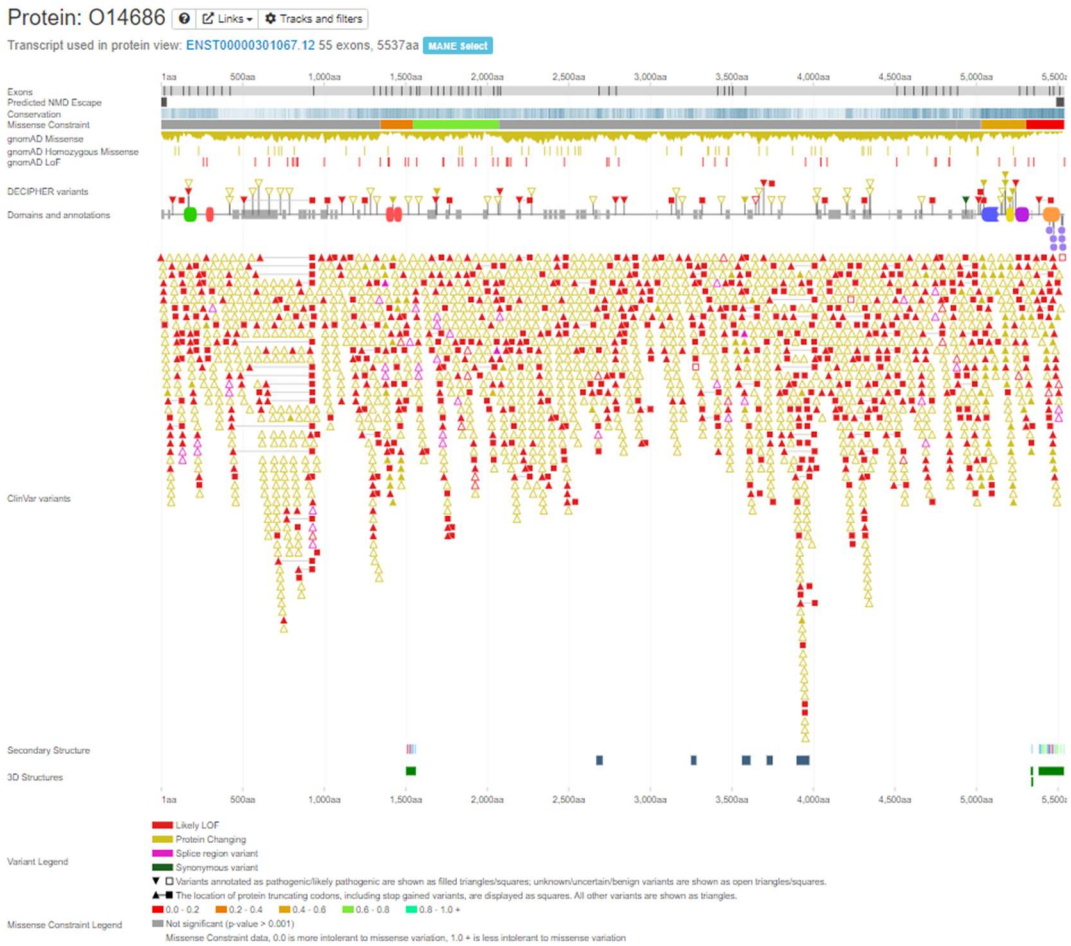



Figure adapted courtesy of Kevin Colclough, Exeter NHS Genomic Laboratory, Royal Devon University Healthcare NHS Foundation Trust; including part of the PVS1 decision tree re-drawn from (Abou Tayoun *et al*., 2018). *Splicing variant should be considered in the context of either: 1) exon skipping, 2) intron retention, 3) use of an alternative splice site. NMD=nonsense mediated decay; NSD = nonstop mediated decay

**Figure 2: Example plot of *KMT2D* from DECIPHER**

Showing functional domains, predicted NMD escape, ClinVar variants, proxy population/benign variants and missense constraint https://www.deciphergenomics.org/




## Copy number variants

The ACMG/ClinGen guidelines have developed a semiquantitative point-based scoring metric for classification of CNVs. Separate scoring metrics have been designed for copy number losses (deletions) and copy number gains (duplications) and these are described in Table 1 and Table 2 respectively of the publication by Riggs *et al*. (2020). The scoring metric ranges from -1.0 to +1.0 points, with positive points being allocated to pathogenic evidence and negative points applied to benign evidence. The point values assigned to each piece of evidence roughly correspond to the categorical strengths of evidence present in the SNV guidelines: evidence receiving (+/-) 0.90 points or higher is considered “very strong"; (+/-) 0.45/0.60 points is considered “strong"; (+/-) 0.30 points is considered “moderate"; and (+/-) 0.15 points or lower is considered “supporting” evidence. These points do not align with the SNV Bayesian evidence point system (Tavtigian *et al*., 2020).

Evidence scores, both in support of and refuting pathogenicity are summed to determine the classification (Table 3). A useful online tool for scoring CNV evidence is available here: https://cnvcalc.clinicalgenome.org/cnvcalc/

**Table 3: Required points values for each CNV classification category**

| Points total | Classification |
|---|---|
| ≥ 0.99 | Pathogenic |
| 0.90 to 0.98 | Likely pathogenic |
| -0.89 to 0.89 | VUS |
| -0.90 to -0.98 | Likely benign |
| ≤ -0.99 | Benign |

A range of points is provided for most scoring categories in the ACMG/ClinGen guidelines to allow flexibility to upgrade or downgrade a certain piece of evidence based on its strength. However, **the default recommended points should be applied** for each piece of evidence in the majority of situations. Examples of when one could default from the recommended score are given below in Table 4. If a decision is made to upgrade or downgrade the points, within the suggested range, for a particular piece of evidence, the points allocated, if you choose to use the evidence (i.e. not apply = 0), should be static: (+/- ) 0.15, 0.30, 0.45/0.60, 0.90/1.00 (these roughly equate to the SNV guidelines strengths of supporting, moderate, strong and very strong). For example, if the default recommended points for a piece of evidence is 0.30 and the range is 0 to 0.45, if you choose to downgrade you would apply 0.15, and if you upgrade would apply 0.45. When the default score of particular evidence is either 0.10 or 0.15 and a range exists, do not upgrade or downgrade the points score.

The ACMG/ClinGen guidelines were developed to be applied to any CNV, irrespective of size or technology used for detection, and unlike the SNV guidelines, the CNV guidelines have standalone evidence for a (likely) pathogenic classification. However, **it is acknowledged that for some CNVs, the use of the SNV guidelines are more applicable**. Therefore, we recommend that these guidelines are applied to the following types of CNVs as stated in **Table 1**:

- the deletion or duplication of a single whole gene
- the partial deletion or duplication of a gene (defined as a CNV that overlaps the 5’ or 3’ end of a gene and contains exon/s)
- the deletion or duplication involving the 5’UTR or 3’UTR only
- the deletion or duplication of multiple contiguous genes

It is recommended that for intragenic CNVs (defined as a CNV with both breakpoints within the gene) involving coding sequence, SNV guidelines should be followed (see **Table 2**).

The scoring metrics were primarily created for the evaluation of autosomal dominant genes/genomic regions of reasonable penetrance. They were not developed with the intent to be independently applied to X-linked genes/regions or those associated with incomplete penetrance and/or variable expressivity; however, many of the concepts are useful for the evaluation of these genes/regions. The scoring metrics were also not created for the evaluation of autosomal recessive genes, but our recommendation is to allow Section 2 to be adapted to

allow for the inclusion of autosomal recessive genes where loss-of-function is an established disease mechanism.

Table 4 contains details from the ACMG/ClinGen guidelines and additional information to help assist with their application. These notes must be used in conjunction with the detailed guidance and tables published by Riggs *et al*. (2020). The guidelines are meant to serve as a guide; professional judgement should always be used when evaluating the evidence surrounding a particular genomic variant and assigning a classification.

**Table 4: Additional information for section usage in CNV guidelines**

| |
|---|
| **Section 1: Initial assessment of genomic content:**<br>This section determines whether the CNV contains protein-coding genes or known functionally important elements. |
| **1A (Table 1 and Table 2)**<br>***Contains protein-coding or other known functionally important elements***<br><br>**1A** (default points = 0)**:** If the CNV contains protein-coding genes, continue with your evaluation. If the CNV does not contain any protein-coding genes*, and is not overlapping population CNVs, it is important to consider the possibility that it contains a functionally important element (which could be a non-coding RNA). If the upstream or downstream flanking genes are within ~1Mb and are associated with a highly specific phenotype which relates to the patient's phenotype, perform a literature search to determine if the region contains a functionally important element for that gene/s (e.g. CNVs within 1Mb of SHH, SOX, HOX and FOX gene families have been reported in the literature). If evidence is found, a classification of likely pathogenic (0.90) can be applied based on the strength of the literature evidence and professional judgement.<br><br>*DECIPHER can be used to list all genes within a CNV and the list can be filtered to display 'protein coding genes'.<br><br>**1B (Table 1 and Table 2)**<br>***Does NOT contain protein-coding or any known functionally important elements***<br><br>**1B** (default points = -0.60)**:** Depending on the method used to detect the CNV, it is important to consider all genes in the maximum breakpoint interval for clinical relevance. If the CNV minimum and maximum interval is intronic but the gene is associated with a highly specific phenotype that fits the patient's clinical presentation and there is literature evidence to suggest the intron is important, then continue with your evaluation. |
| **Section 2: Overlap with Established (or Predicted) Pathogenic or Benign Dosage Sensitive Genes/Genomic Regions:**<br>In this section, the CNV is evaluated for overlap with established dosage sensitive genes or genomic regions or established benign genes or genomic regions.<br><br>*If a score of ≥0.99 (Pathogenic) is reached in this section, application of subsequent sections is not required.* |
| Various curations are used to help determine if a gene/region is dosage sensitive (https://www.clinicalgenome.org/site/assets/files/6428/dosage_sop-scoring-1.pdf, https://www.ebi.ac.uk/gene2phenotype, https://clinicalgenome.org/site/assets/files/5391/version_9_gene_curation_sop_final2.pdf).<br><br>To be considered an established pathogenic dosage sensitive gene/region it will have one or more of the below:<br>• ClinGen Dosage Sensitivity haploinsufficiency (HI) score of 3 ('sufficient evidence')<br>• Monoallelic Gene2Phenotype (G2P) gene with a 'definitive' or 'strong' status and 'absent gene product' as the consequence<br>• Biallelic G2P gene with a 'definitive' or 'strong' status and 'absent gene product' as the consequence (see below) |

- Gene-Disease Validity (ClinGen) with a 'definitive' or 'strong' status and sufficient evidence of predicted or proven null variants to show loss-of -function is a disease mechanism (either AD or AR genes)
- ClinGen Dosage sensitivity triplosensitivity (TS) score of 3 ('sufficient evidence')

In general, if the gene/region has already been curated as above, it is not essential to perform a formal curation (like the framework developed by (Strande *et al*., 2017), but laboratories are expected to ensure that the evidence provided for the curations was sufficient for the gene/disease association and that loss-of-function is the mechanism of disease.

*Please note:*

- Some genes will only have had loss-of-function sequence variants reported previously. However, it is acceptable to expect a copy number loss to have the same consequence.
- When reviewing ClinGen DS scores, it is important to note the date of last evaluation, as curations reflect a temporally static assessment. New evidence may have emerged since the date of last evaluation, either supporting or refuting the original assessment.
- Genes with a ClinGen Dosage haploinsufficiency score of 2 (i.e. 'emerging evidence' of HI) or a G2P status of 'moderate' and 'absent gene product' are likely to have enough available evidence to reach a classification of at least likely pathogenic but scrutiny of the evidence and application of the guidelines is still required.
- Alternative transcripts to the MANE Select transcript should only be considered in the upgrade or downgrade of points in Section 2 if the alternative transcript is a MANE Plus Clinical transcript or considered the most biologically expressed transcript.

To be considered an established benign gene/region it will have one or more of the below:

- ClinGen Dosage sensitivity score of 40 ('dosage sensitivity unlikely')
- Commonly seen CNV within cohort that has a platform frequency of >1%
- A frequency >1% on the DGV gold standard dataset or gnomAD SV dataset

When using reported frequency from population databases you must consider the size/number of alleles examined within the study/s used. Some frequencies can be skewed if the variant was only examined in a small proportion of cases. As a general rule, it is recommended that frequency calculations are based on a minimum of 10,000 alleles/5000 samples. The gnomAD SV v4.0 dataset contains 63,046 genomes; the sample size of studies used in the DGV database can be found here: http://dgv.tcag.ca/dgv/app/search?ref=GRCh37/hg19#tabs-view_all_info_study

**2A + 2B (Table 1 and Table 2) and 2H (Table 2)**

***Complete overlap with an established HI/LOF or TS gene/region + partial overlap of an established HI/LOF or TS region***

**2A** (default points = 1.00)**:** CNVs completely containing established dosage sensitive genes/regions are a pathogenic classification and do not require any further evidence to be gathered; however, you may still need to investigate the clinical implications of the CNV and its association with your patient's phenotype/management for the report.

**2B** (default points = 0)**:** CNVs that partially overlap established dosage sensitive regions where the critical gene/region has not yet been established require further investigations.

**2H (Table 2)** (default points = 0)**:** An established HI/LOF gene that is fully contained within a duplication should not be presumed to be disease-causing unless the gene is also known to be associated with a triplosensitive disorder.

**2C – 2D (Table 1) and 2J – 2K (Table 2)**

***Partial overlap with an established HI/LOF (one breakpoint is within the gene and the other not)***

If the CNV partially overlaps the 5' or 3' end of a curated HI/LOF gene, additional evaluation to determine the potential functional effect is required based on breakpoint location, involvement of coding sequence and evidence from the literature.

**Table 1 (2C):** If the 5' end of the gene is involved, you must consider whether the deletion also involves additional coding sequence:

**2C-1** (default points = 0.90; range = 0.45 to 1.00)**:** Deletions that include 5'UTR (untranslated region), first coding exons, or internal exons are typically deleterious in HI/LOF genes, the recommended default score is 0.90 points.

- If there is another potential in-frame methionine (start codon) downstream, downgrade the default points from 0.90 to 0.45.
- If a significant portion of the coding sequence is deleted, or if a known functionally important domain is deleted, consider upgrading the default points to 1.00.

**2C-2** (default points = 0; range = 0 to 0.45)**:** When only the 5'UTR is involved, the recommended default score is 0 points.

- If there is evidence to support the role of the non-coding 5'UTR region in disease, upgrade the suggested default number of points to 0.45.

**Table 1 (2D):** If the 3' end of the gene is involved, you must consider whether the resulting protein product is expected to undergo nonsense-mediated decay – NMD. Deletions that involve the last exon of a HI/LOF gene are not always pathogenic unless affecting a key functional domain.

**2D-1** (default points = 0)**:** When only the non-coding 3'UTR is involved, the recommended default score is 0 points because the resulting protein product is expected to escape NMD.

**2D-2** (default points = 0.90; range = 0.45 to 0.90)**:** If the deletion involves only the last exon, the resulting protein product is expected to escape NMD. But if other established pathogenic variants have been reported in the last exon then such a deletion may be disease-causing. The default score is 0.90 points but you must be confident that the last exon is important and critical to gene function (e.g. documented pathogenic variants, functional studies showing that loss of the last exon results in disrupted function, the last exon is within an established variant hotspot).

- If there are pathogenic variants reported involving only the last exon but it has not been established fully that the exon is important, downgrade the default points to 0.45 or 0.60 depending on the level of confidence that the last exon is critical to gene function.

**2D-3** (default points = 0.30; range = 0 to 0.45)**:** If the deletion involves only the last exon, but there is no evidence to suggest that the last exon is critical to the gene function (e.g. no other pathogenic variants have been reported in that exon) the recommended default score is 0.30.

**2D-4** (default points = 0.90; range = 0.45 to 1.00)**:** If the deletion overlaps the 3' end of the gene and includes exons other than the last exon, NMD is expected to occur, the recommended default score is 0.90.

- If a significant percentage of the protein is expected to be missing, upgrade from the default score to 1.00 points.

**Table 2 (2J – 2L):** Partial overlapping duplications involving the 5' or 3' end of a gene are typically expected to be in tandem and in direct orientation (Newman *et al*., 2015) and not deleterious because functional gene structure may be preserved. *If* the variant is causing an impact on the gene, it would be expected to be a HI/LOF effect; therefore, you should be assessing partial gene duplications against HI/LOF and not TS. The allocation of any points to such variants will be rare; it requires the gene to be an established HI/LOF disease-associated gene and also requires your patient's phenotype to be *highly specific* to the phenotype associated with the gene.

**2J** (default points = 0)**:** If the patient's phenotype is not consistent with that expected from HI/LOF of the gene, continue with your evaluation.

**2K** (default points = 0.45)**:** If the patient's phenotype is highly specific and consistent with that expected from LOF of the gene.

- There is no points range for this category of evidence. Again, this reflects the fact that most partial-gene duplications are not disruptive (in tandem and direct orientation). The requirement of the patient's phenotype to be *highly specific* to that expected from HI/LOF of the gene will make it difficult to apply this category to any partial duplication involving a gene associated with a non-specific phenotype.
- If the duplication is proven to be in tandem and in direct orientation, 2K should not be applied.

**2L (Table 2)**

***Partial overlap with a gene with no known disease association (one breakpoint is within the gene and the other not)***

**2L** (default points = 0)**:** If the partial duplication involves a gene that is not an established HI/LOF disease-causing gene then no points are applied, and you continue with your evaluation. Applies also to when both breakpoints are within a gene with no disease association.

**2E (Table 1) and 2I (Table 2)**

***Intragenic del/dup within established HI/LOF gene (both breakpoints within the gene)***

Use the SNV guidelines for all intragenic CNVs.

**2H (Table 1)**

***≥2 HI predictors suggest at least 1 gene in the interval is HI***

**2H** (default points = 0.15)**:** Predictive *in silico* scores for HI of genes can be found on the DECIPHER database under the gene entry.

Common HI predictors that can be applied to this category include:

- pLI, LOEUF, sHET and pHaplo (definitions, thresholds + information can be found on DECIPHER)
    - please note that pLI and LOEUF use the same gnomAD data but presented differently and should not be used together.
    - %HI scores have been removed from DECIPHER and been replaced by pHaplo.

Do not use for deletions that involve an established HI/LOF gene (i.e. if you have already applied categories 2A-2D-4, Table 1). The points associated with 2H should only be given once; do not count this piece of evidence multiple times, even if there is more than one gene in the region predicted to be HI.

**2F + 2G (Table 1) and 2C-2G (Table 2)**

***Identical or completely contained within or overlaps with an established benign region***

**2F (Table 1); 2C, 2D, and 2F (Table 2)** (default points = -1.00): When a CNV under evaluation is contained completely within an established benign region or is larger but contains no additional genes.

- If your CNV is a partially overlapping gene duplication you cannot use established benign regions that encompass the whole of that gene in your evaluation. The expected potential impact of intragenic or partial gains is different to that of a whole gene duplication. In such situations it may be more applicable to compare your CNV to established benign copy number losses.

- Be aware that some autosomal recessive variants could have a population frequency >1% and should not be interpreted as benign based solely on their frequency.

**2G (Table 1 and Table 2)** (default points = 0): If the CNV only overlaps the benign region, or is larger, and contains additional genes, you must continue your evaluation to determine if the region contains any clinically relevant genes or functionally important elements.

**Section 3: Evaluation of Gene Number**

This section evaluates the number of protein coding genes involved in the CNV.

**3A – 3C (Table 1 and Table 2)**

***The number of genes wholly or partially involved in the CNV***

In general, when a CNV contains a very large number of genes, it becomes more likely that loss or gain of this amount of genetic material will result in some demonstrable phenotypic consequence.

**3A** (default points = 0)**:** Deletions containing less than 24 genes and duplications containing less than 34 genes.

**3B** (default points = 0.45)**:** Deletions containing between 25 and 34 genes, or duplications containing between 35-49 genes.

**3C** (default points = 0.90)**:** Deletions containing 35 or more genes, or duplications containing 50 or more genes.

Caution should be applied if the CNV involves clusters of genes or gene families, particularly those that are non-coding or lack any known clinical association. Each cluster/family should be counted as a single gene. If a gene within a cluster/gene family has an OMIM Morbid entry, count them as an individual gene.

**Section 4: Detailed Evaluation of Genomic Content Using Cases from Published Literature, Public Databases, and/or Internal Laboratory Data**

In this section evidence is gathered of comparable cases in the literature and databases to support or refute the clinical significance of the gene/genomic region under evaluation.

*If you have reached a score of ≥0.99 (Pathogenic) you can skip this section.*

The nature of CNVs mean that their evaluation often requires the assessment of both genes of known and unknown clinical significance. Many CNVs will not overlap *established* dosage sensitive or benign genes/regions. These CNVs require evaluation using the literature and databases to establish if a similar CNV has been reported in another proband/family and whether or not the CNV has a clinical association.

Section 4 (Table 1 and Table 2) is split into two evidence types:

1. **Individual Case Evidence (4A-4K)**
2. **Case-Control or General Population Data (4L-4O)**

### 1. Individual Case Evidence

**4A – 4E (Table 1 and Table 2)**

***Assessment of the literature and databases for similar CNVs to your region of interest***

**4A-4C:** Evaluates *de novo* case evidence by determining how consistent the reported phenotype in the similar case/s is to what is expected for that gene/region, how specific that phenotype is, how unique it is to the gene/region, and whether the *de novo* status is confirmed or assumed*.*

Cases in the literature/databases with highly specific, well-defined phenotypes, that have been confirmed to have arisen *de novo* (e.g. trio genome sequencing has been undertaken – which will confirm maternity and paternity), represent the strongest evidence. The less specific the reported phenotype is, the less certain you can be that it is related to your gene/region of interest and not to other genes/factors. Also, assumed *de novo* (e.g. parents tested on microarray) is associated with fewer points consistent with the approach taken in the SNV guidelines.

"Highly specific, well-defined" phenotypes have a distinct and known genetic aetiology with limited genetic heterogeneity. "Non-specific" phenotypes are those that may be more common, have more considerable genetic heterogeneity, and/or can be caused by aetiologies other than genetic variation.

| **Phenotype** | **Examples** |
|---|---|
| Highly specific, relatively unique to the gene/region | Fixed dilated pupils (Gillespie syndrome)<br>Fetal adrenocortical cytomegaly (Beckwith-Wiedemann syndrome)<br><br>Specific scan findings, biochemical assay results etc |
| Highly specific, not necessarily unique to the gene/region | Leukodystrophy<br>Metopic ridging<br>Skeletal dysplasias<br>EIEE (not simply 'seizures' or 'epilepsy') |

| | |
|---|---|
| | Constellation of findings like coloboma, heart defects, choanal atresia, growth delays, genital anomalies, and ear abnormalities associated with CHARGE syndrome |
| Not highly specific, and/or with high genetic heterogeneity | Intellectual disability<br>Developmental delay<br>Autism |

Functional studies (e.g. *in vitro* or *in vivo* animal model studies, biochemical assays, or scan findings etc) may support the specificity of the reported phenotype and therefore determine which evidence category to use; they should not be used to upgrade the points value for a particular category.

A range of points is provided for 4A-4C. Use the default number of points for each case or none at all if the evidence is weak. For example:

- **4A** will be either 0.45 (confirmed *de novo*) or 0.30 (assumed *de novo*)
- **4B** will be either 0.30 (confirmed *de novo*) or 0.15 (assumed *de novo*)
- **4C** will be either 0.15 (confirmed *de novo*) or 0.10 (assumed *de novo*)

Note that the maximum total score you can reach using evidence from cases that 4A – 4C is applicable to is 0.90.

**4D** (default points = 0; range = 0 to -0.30)**:** For *de novo* cases in which the reported phenotype is either not consistent with what is expected for a given gene/region, or simply not consistent in general, the recommended points applied is 0.

- For negative points to be applied to cases using 4D, clinical judgement should be used to determine if there is strong enough evidence of inconsistency in phenotypes across the cases and if appropriate phenotyping has been undertaken and if the cases are comparable. However, it is unlikely that sufficient evidence will be found to award negative points.
- If evidence is found, apply -0.15 points to each case.

Note that the maximum score you can reach using evidence from cases that 4D is applicable to is -0.30.

**4E** (default points = 0.10; max. total = 0.30)**:** For cases where the inheritance is unknown, points can only be applied to each case if the phenotype is *highly specific* and consistent with that expected for the gene/region.

**Genes of unknown clinical significance:** When the gene(s) being assessed are of unknown clinical significance there must be at least two *de novo* cases with similar phenotypes before points can be assigned (i.e. the two cases count as one piece of evidence/case). Each additional case observed with a consistent phenotype may be awarded additional points. If only a single *de novo* case has been reported in the literature/databases, and the variant under evaluation by the laboratory is *de novo* with a phenotype consistent with the phenotype of the single reported case, then both cases can be used together as a single piece of evidence and the appropriate category can be applied. In this scenario, additional points should not be assigned to your case in Section 5.

- Use the points values from 4A – 4C for your evidence. For example, if the similar phenotype is highly specific and confirmed *de novo* status, 0.45 points is applicable; if the similar phenotype is non-specific and the cases are assumed *de novo* then only 0.10 points is applicable.

**4F – 4K (Table 1 and Table 2)**

***Assesses the segregation of the similar CNV within families in the literature and databases.***

Segregation of a variant among similarly affected family members can lend support to the argument that the variant may be disease-causing. Instances in which the variant appears not to segregate with affected status does not automatically mean it is not disease-causing.

**4F-4H:** Covers segregation among similarly affected family members. The CNV guidelines adopt a conservative approach to segregation because segregation implicates a locus, and not necessarily a particular gene or variant, and because of the difficulty in determining whether CNVs reported in the literature/databases are the same given the variability in the accuracy of breakpoints depending on the technology used. Therefore at least 3 documented segregations among affected individuals are required in order to assign any points:

- **4F** (default points = 0.15): 3-4 segregations
- **4G** (default points = 0.30): 5-6 segregations
- **4H** (default points = 0.45): 7 or more segregations

Only those individuals with both the genotype and the phenotype, or individuals who are obligate carriers, can be counted as evidence. Segregations can be added across families. When counting segregations, the proband is not counted:

- # of segregations = (# of genotype/phenotype positive) – 1

Caution should be applied when using large families for segregation analysis. The maximum number of points allocated to CNV segregation from the literature and databases is 0.45. The guidelines aim to avoid situations where a CNV could reach a likely pathogenic/pathogenic classification by simply using evidence of segregation from one family. The goal is to try and collect as many diverse pieces of information as possible to determine the classification of the variant. However, for some variants this is not possible, and if there is substantial segregation evidence, overriding the maximum points could be considered (e.g., well-studied gene with definitive gene-disease association and numerous unrelated families with documented segregation).

*Please note:*

The CNV guidelines separate literature/database segregation (Section 4F-4H) from your case segregation (Section 5D), whereas the SNV guidelines incorporate them together. Points collated for segregation evidence gathered from both sections can be used towards the classification of the variant e.g., 4H at 0.45 plus 5D(~4H) at 0.45 = 0.90 points.

**4I** (default points = -0.45; max. total = -0.90)**:** Apparent non-segregations include instances in which another *affected* individual in the case family is found *not* to have the variant in question. Assign the default number of negative points when the individuals in the family are affected with similar, *highly specific* phenotypes (with no known phenocopies) and are not found to carry the same variant.

- Consider downgrading this evidence to -0.15 (supporting) when phenocopies are a possibility.

**4J and 4K:** Instances in which an *unaffected* individual *is* found to have the variant in question, the specificity of the phenotype in the affected family member/s plays a role in how many points can be applied.

- **4J** (default points = -0.30; max. total = -0.90): specific, well-defined phenotype
- **4K** (default points = -0.15; max. total = -0.30): nonspecific phenotype

Consider whether the family member found to have the variant is truly unaffected. Do not assign negative points if there's a plausible explanation why the phenotype may not be present/observed/reported (e.g. known incomplete or age-related penetrance, clinical feature not readily observable, individual not properly evaluated, variable expressivity), or if the family member is simply stated in a publication/database not to be affected, but no details of their evaluation are provided. If the individual has been clinically phenotyped or tested for the particular phenotypic feature (e.g. by radiological investigation or biochemical assay), and there is no evidence of the phenotype, assign the default number of negative points. Note that phenotypes segregating in a family may exhibit variable expressivity but may still be part of the same phenotypic spectrum (e.g. autism spectrum disorder, intellectual disability, seizures, and schizophrenia observed in different individuals may all be caused by the same CNV as part of the developmental brain disorder spectrum).

### 2. Case-Control or General Population Data

**4L, 4M, 4N (Table 1 & Table 2)**

***CNV has been studied as part of a case-control study***

If the CNV has been studied as part of a well-powered case-control study, points may be added or deducted based on enrichment (or lack thereof) in the clinical population. Interpretation of case-control data should include evaluation of significance (i.e. p-value), effect size (e.g. likelihood ratio), and clinical information (e.g. phenotypic specificity). CNVs in this category will be observed at a significantly higher frequency in cases versus controls ($p \leq 0.05$), and with a strong effect size (odds ratio or likelihood ratio >5) and relatively narrow associated 95% confidence interval (lower bound >1).

CNVs with the highest points values will be observed in association with a consistent, specific, well-defined phenotype (0.45 points, **4L**). Those lacking phenotypic specificity, but enriched, can also be counted (0.30 points,

**4M**). CNVs that are not enriched and are observed at similar (or higher) frequencies in controls compared to cases, can be deducted points (-0.90 points, **4N**).

Case-control study data is rarely available for rare diseases. However, in-house datasets (case cohort) and the gnomAD-SV v4 and gnomAD-CNV v4 datasets (control cohort) can be used to calculate a p-value using https://www.medcalc.org/calc/odds_ratio.php to allow 4L to be applied at reduced points if the variant has been identified in multiple (two or more) unrelated affected individuals (with a consistent, **rare,** well-defined phenotype). It is acceptable to apply 4L at 0.30 if the variant has a p-value of ≤0.05 and at 0.15 points if the variant has a p-value of ≤0.1. Case-control data ideally needs to be from equivalent ethnic groups.

**4O (Table 1 & Table 2)**

***CNV is present in population databases***

**4O** (default points = -1.00; range = 0 to -1.00): This category covers CNVs that involve regions seen in population databases. There is overlap between the evidence that can be used for categories 4O and that used for 2F (Table 1) and 2C, 2D, and 2F (Table 2) for established benign variants (frequency >1%). Category 4O is used for variants that are present at a frequency less than 1%:

| Frequency | Points |
|---|---|
| >0.5% | -0.90 |
| 0.1-0.5% | -0.30 |
| 0.01-0.09% | -0.15 |

## Section 5: Evaluation of Inheritance Pattern/Family History for your Patient

This section evaluates the inheritance and family history of the CNV being studied

Your patient's phenotype is incorporated into Section 5, which covers the inheritance pattern/family history for your case. The points assigned to inheritance information in this section (*de novo*, inherited from an unaffected family member, inherited from an affected family member) is modified by the specificity of your patient's phenotype. The points-metric is based around how specific and well-defined the patient's phenotype is in relation to the gene/region in question and follows the principles laid out in Section 4; highly specific, well-defined phenotypes represent stronger evidence and more points than disparate or non-specific phenotypes.

**5A (Table 1 and Table 2)**

***The CNV in your case is found to be de novo***

**5A:** Use the scoring metrics from 4A-4D. If your patient's phenotype is:

- highly specific and relatively unique to the gene/region (~4A)
  - apply 0.45 points (if confirmed *de novo*)
  - apply 0.30 points (if assumed *de novo*)
- consistent with the gene/region, is highly specific but not necessarily unique to the gene/region (~4B)
  - apply 0.30 points (if confirmed *de novo*)
  - apply 0.15 points (if assumed *de novo*)
- consistent with the gene/region, but not highly specific and/or with high genetic heterogeneity (~4C)
  - apply 0.15 points (if confirmed *de novo*)
  - apply 0.10 points (if assumed *de novo*)
- not consistent with the gene/region (~4D)
  - apply 0 points

**5B – 5D (Table 1 and Table 2)**

***The CNV in your case is found to be inherited***

**5B** (default points = -0.30)**:** Apply if your patient's phenotype is *specific and well-defined* and has been inherited from an unaffected parent.

**5C** (default points = -0.15)**:** Apply if your patient's phenotype is *non-specific* and has been inherited from an unaffected parent.

**5D:** Use the scoring metrics from 4F-4H. If your patient's CNV is segregating with a consistent phenotype in the family and the number of segregations are:

- 3-4 segregations (~4F)
    - apply 0.15 points
- 5-6 segregations (~4G)
    - apply 0.30 points
- 7 or more segregations (~4H)
    - apply 0.45 points

**5E (Table 1 and Table 2)**

***The CNV in your case is not segregating in the family***

**5E:** Use the scoring metrics from 4I-4K. If your patient's CNV is:

- not found in another family member affected with a consistent, specific, well-defined phenotype (~4I)
    - apply -0.45 points
- found in another family member unaffected with the specific, well-defined phenotype (~4J)
    - apply -0.30 points
- found in another family member unaffected with the non-specific phenotype (~4K)
    - apply -0.15 points

**5F – 5H (Table 1 and Table 2)**

***The inheritance information for the CNV is unknown***

If inheritance studies have not been undertaken or are uninformative (e.g. only one parent received), no points are applied **(5F)**.

However, if the patient's phenotype in its entirety is consistent with a specific genetic aetiology, points may be assigned **(5G and 5H)**. These categories should be considered equivalent to using PP4 in the sequence variant guidelines at supporting or moderate strength.

**5G** (default points = 0.10, Table 1 and Table 2)**:** Can be applied if your patient's phenotype is non-specific but is consistent with what has been described in other similar cases.

- 5G should not be applied simply because the patient has a non-specific phenotype such as intellectual disability or autism spectrum disorder. Your patient must have multiple non-specific features that are the same as described in other similar cases.

**5H** (default points = 0.30, Table 1; default points = 0.15 points, Table 2)**:** Can be applied if your patient's phenotype is highly specific and consistent with what has been described in other similar cases.

A range of points exists for 5G and 5H but the default points should always be applied if the evidence is used.

## 6. Classifying variants in the mitochondrial genome

The increased use of whole genome sequencing in rare disease diagnosis has increased the likelihood of uncovering rare sequence variants in the mitochondrial genome (mtDNA). The evaluation of pathogenicity of mtDNA variants differs substantially from those detected in the

nuclear genome, with additional considerations regarding levels of variant heteroplasmy, the tissue tested, mtDNA haplogroup and reliable frequency resources. We recommend that laboratories unfamiliar with mtDNA analysis seek support from specialist mitochondrial centres in more challenging cases, and that the current ACGS Best Practice Guidelines for the Molecular Diagnosis of Mitochondrial Disease (https://www.acgs.uk.com/media/11935/bpg-for-the-molecular-diagnosis-of-mitochondrial-disease_ratified-november-2020.pdf) (Mavraki *et al*., 2023) (McCormick *et al*., 2020) are followed.

## 7. Reporting the variant classification

Variants are classified as “pathogenic”, “likely pathogenic”, “uncertain significance”, “likely benign” or “benign” with respect to a disease and inheritance pattern. The evidence and hence variant classification are dependent upon knowledge at the time of the assessment, and it is important that service users understand that new information or guidelines may change the classification.

The aim of genomic testing for a patient with a rare disease of unknown cause is to provide a genetic diagnosis by identifying (likely) disease-causing variant(s) that explain the clinical presentation. The genetic analysis may involve the classification of one or multiple variants, but the genomic laboratory report and any appendices should only describe those that are relevant, or have likely relevance, to the clinical question being addressed by the test i.e. VUS with high probability of being causative, likely pathogenic or pathogenic variants. Results included within the genomic laboratory report will form part of the patient’s clinical record and should be unambiguous to a non-specialist. In the situation that the testing does not identify (likely) disease-causing variant(s), the report should clearly state that the result does not exclude a genetic diagnosis.

Variants should be reported using ISCN or HGVS nomenclature and include a clear description of the variant in words in the main report text e.g. pathogenic missense variant, copy number loss involving exons 2-6, copy number gain of 113 OMIM morbid genes etc. Reports must include the human reference genome build and where applicable the clinically appropriate transcript and version number (e.g. MANE select and/or MANE clinical plus). It is more appropriate to use the terms (copy number) loss and (copy number) gain, to describe the genomic imbalance. The terms ‘deletion’ and ‘duplication’ should only be used when the underlying mechanism of the abnormality is known i.e. recurrent CNVs generated by non-allelic homologous recombination (NAHR). The genotype of the variant should be included in its description and where applicable reports should define the position of variants (e.g. interstitial, terminal, tandem, inverted).

For intragenic CNVs, it is crucial that the description should be unambiguous, ideally including the systematic numbering of impacted exons in a specific named reference sequence (“exons 1-3 in NM_000251.3”), and ideally, either precise HGVS describing the variant (where this is known) or approximate genomic coordinates of the deletion based on limitations of the assay used (e.g. MLPA).

The variant classification must be included within the results section of the genomic laboratory report together with clear information regarding the gene-disease association and the mode of inheritance. We ***recommend*** that the evidence supporting the variant classification is included

in an appendix to the report for reportable VUS and likely pathogenic variants. Variants classified as likely benign or benign should not be reported.

**Example NHS reports are available on the members area of the ACGS website https://www.acgs.uk.com/ and https://www.cangene-canvaruk.org/canvig-uk-report-templates.**

In **Table A** we summarise our recommendations for reporting genomic variants. This table describes which variants to include in the genomic laboratory report, text that should be used within the result summary box and some explanatory notes. Additional specific notes for recessive disorders are provided in **Table B**.

**Table A: Recommended approach to reporting genomic variants in probands**

Note from (Richards *et al.*, 2015) that pathogenic is proposed to mean a 99% certainty that the variant is disease-causing and likely pathogenic equates to 90% certainty.

| Classification | Variant included in report? | Result summary for genomic laboratory report | Explanatory notes |
|---|---|---|---|
| **Pathogenic** | Yes | **Genetic diagnosis of disorder/syndrome X**<br><br>OR<br><br>**Genetic diagnosis of (syndromic) intellectual disability**<br><br>OR<br><br>**Genetic diagnosis of (“*Gene name”)* - related disorder**<br><br>OR<br><br>**Confirms a genetic diagnosis of disorder X** (if there was clinical suspicion of this specific disorder)<br><br>OR<br><br>**Pathogenic unbalanced CNV detected** | Very high likelihood that the variant is causative of the disorder<br><br>*>99% certainty that the variant is pathogenic* |

| | | | |
|---|---|---|---|
| **Likely pathogenic** | Yes | **Consistent with a genetic diagnosis of disorder/syndrome X**<br><br>OR<br><br>**Consistent with a genetic diagnosis of (syndromic) intellectual disability**<br><br>OR<br><br>**Consistent with a genetic diagnosis of ("*Gene name")* - related disorder**<br><br>OR<br><br>**Likely pathogenic CNV detected** | High likelihood that the variant is causative of the disorder<br><br>*>90% certainty that the variant is pathogenic* |
| **Uncertain significance***<br><br>*VUS where further testing or investigations could be considered as the results have the potential to change the classification to likely pathogenic* | Yes | **Variant of uncertain significance identified – consider further action**<br><br>Clearly describe in the report the action required that could change the classification to likely pathogenic | Further testing or investigations could be undertaken to re-classify the variant as likely pathogenic.<br><br>Examples include:<br>a) testing parents to determine whether *de novo*<br>b) mRNA analysis for variants predicted to cause aberrant splicing<br>c) testing affected relatives to show co-segregation<br>d) Clinical assessment and/or investigation(s) to provide phenotypic specificity evidence<br>e) trial of a treatment that is specific for the genetic aetiology<br>f) determining the location and/or orientation of copy number gains |

| | | | |
|---|---|---|---|
| **Uncertain significance***<br><br>*VUS where no further evidence can be obtained* | Not usually | **A genetic cause for the patient's clinical presentation has not been identified**<br><br>State in the result text that the result does not exclude a genetic diagnosis | These variants should only be reported in exceptional circumstances following MDT discussion (see section 7.1.1). |
| **Uncertain significance***<br><br>*VUS where no further evidence can be obtained* | No | **A genetic cause for the patient's clinical presentation has not been identified**<br><br>State in the result text that the result does not exclude a genetic diagnosis | These variants are almost invariably unlikely to be disease-causing and are potentially confusing if included in the report. They should only be reported in exceptional circumstances following MDT discussion (see section 7.1.1). |
| **Likely benign** | No | **A genetic cause for the patient's clinical presentation has not been identified**<br><br>State in the result text that the result does not exclude a genetic diagnosis | These variants are not relevant and potentially confusing if included in the report<br><br>*>90% certainty that the variant is benign* |
| **Benign** | No | **A genetic cause for the patient's clinical presentation has not been identified**<br><br>State in the result text that the result does not exclude a genetic diagnosis | These variants are not relevant and potentially confusing if included in the report<br><br>*>99.9% certainty that the variant is benign* |
| *Variant previously reported in the literature as (likely) pathogenic but now classified as (likely) benign* | No | **A genetic cause for the patient's clinical presentation has not been identified**<br><br>State in the result text that the result does not exclude a genetic diagnosis | There is no clinical utility in reporting these historical false positive results and potential risk of misinterpretation |
| *A variant type/mechanism that does not fit with the established disease mechanism* | No | **A genetic cause for the patient's clinical presentation has not been identified**<br><br>State in the result text that the result does not exclude a genetic diagnosis | For example a loss of function variant in a gene where the disease mechanism is gain of function or is mediated via an effect upon a specific protein structure |

***See Figure 3 for sub-classifications of variants of uncertain significance**

**Table B: Additional recommendations for reporting genomic variants in recessive disorders**

| Classification | Variant(s) included in report? | Result summary for genomic laboratory report | Explanatory notes |
|---|---|---|---|
| *Recessive disorder with two pathogenic variants identified and known to be in trans* | Yes | **Genetic diagnosis of disorder X**<br><br>OR<br><br>**Genetic diagnosis of (“*Gene name*”)-related disorder**<br><br>OR<br><br>**Confirms a genetic diagnosis of disorder X** (if cases where there was clinical suspicion of this specific disorder) | Very high likelihood that the variants are causative of the disorder<br><br>*>99% certainty that each variant is pathogenic* |
| *Recessive disorder with one pathogenic and one likely pathogenic variant identified and known to be in trans* | Yes | **Consistent with a genetic diagnosis of disorder X**<br><br>OR<br><br>**Consistent with a genetic diagnosis of (“*Gene name*”)-related disorder** | High likelihood that the variants are causative of the disorder<br><br>*>90% certainty that each variant is pathogenic* |
| *Recessive disorder with two likely pathogenic variants identified and known to be in trans* | Yes | **Consistent with a genetic diagnosis of disorder X**<br><br>OR<br><br>**Consistent with a genetic diagnosis of (“*Gene name*”)-related disorder** | High likelihood that the variants are causative of the disorder<br><br>*>90% certainty that each variant is pathogenic* |
| *Recessive disorder with two pathogenic variants identified where not known if variants are in trans* | Yes | **Consistent with a genetic diagnosis of disorder X**<br><br>OR<br><br>**Consistent with a genetic diagnosis of (“*Gene name*”)-related disorder** | High likelihood that the variants are causative of the disorder<br><br>*>99% certainty that each variant is pathogenic* |
| *Recessive disorder with one pathogenic and one likely pathogenic variant identified* | Yes | **Consistent with a genetic diagnosis of disorder X – parental testing required** | Likely that the variants are causative of the disorder |

| | | | |
|---|---|---|---|
| *where not known if variants are in trans* | | | *>90% certainty that each variant is pathogenic* |
| *Recessive disorder with two likely pathogenic variants identified where not known if variants are in trans* | Yes | **Possible genetic diagnosis of disorder X – parental testing required** | Likely that the variants are causative of the disorder<br><br>*>90% certainty that each variant is pathogenic* |
| *Recessive disorder with one (likely) pathogenic variant and a reportable VUS** | Yes | **Possible genetic diagnosis of disorder X – additional evidence required to clarify this result** | When it is not possible to obtain sufficient evidence to classify the VUS as likely pathogenic but all the available clinical, gene-level and variant-level evidence supports the likely diagnosis |
| *Recessive disorder with homozygous reportable VUS** | Yes if appropriate e.g. parents are consanguineous and phenotype is consistent with gene | **Variant of uncertain significance identified – consider further action**<br><br><u>Clearly</u> describe in the report the action required that could change the classification to likely pathogenic | Recommend further testing or investigations that could be undertaken in order to reclassify the variant as likely pathogenic. |
| *Recessive disorder with one (likely) pathogenic variant* | Only appropriate in specific circumstances e.g. patient's phenotype highly consistent with disorder; consanguineous family; high carrier freq and would affect reproductive/clinical management | **See section 7.2** | Either an incidental carrier finding or a second (likely) pathogenic variant has not been detected and further genetic testing is required. |

| | | | |
|---|---|---|---|
| *Recessive disorder with one VUS* identified* | No | **A genetic cause for the patient's clinical presentation has not been identified**<br><br>State in the result text that the result does not exclude a genetic diagnosis | There is no clinical utility in reporting a single heterozygous VUS with potential risk of misinterpretation |

***See Figure 3 for sub-classifications of variants of uncertain significance**

All germline CNVs should be interpreted and assigned a classification in accordance with these guidelines; this includes CNVs detected in a postnatal, prenatal and pregnancy loss setting. The classification of a CNV should be consistent irrespective of the clinical setting, however the decision on whether to report certain CNVs in a prenatal or pregnancy loss setting may vary depending on the clinical presentation.

Many recurrent dosage sensitive regions and genes have been curated by the ClinGen Dosage Sensitivity curation group (https://www.clinicalgenome.org/, therefore to help with the standardisation of the classification of CNVs involving these regions/genes, which are frequently detected in copy number analysis, we recommend the following: those that have a haploinsufficiency/triplosensitivity score of 3 (sufficient evidence) equates to a Pathogenic classification, a score of 2 (emerging evidence) equates to Likely Pathogenic, and a score of 1 (little evidence) equates to a VUS. In accordance with the guidance for the reporting of variants of uncertain significance (see section 7.1), we do not recommend the reporting of recurrent regions/genes that have been assigned a VUS classification. Those that have a score of "40" (dosage sensitivity unlikely) equates to a Benign classification. It is important to check the curations to ensure they have remained consistent.

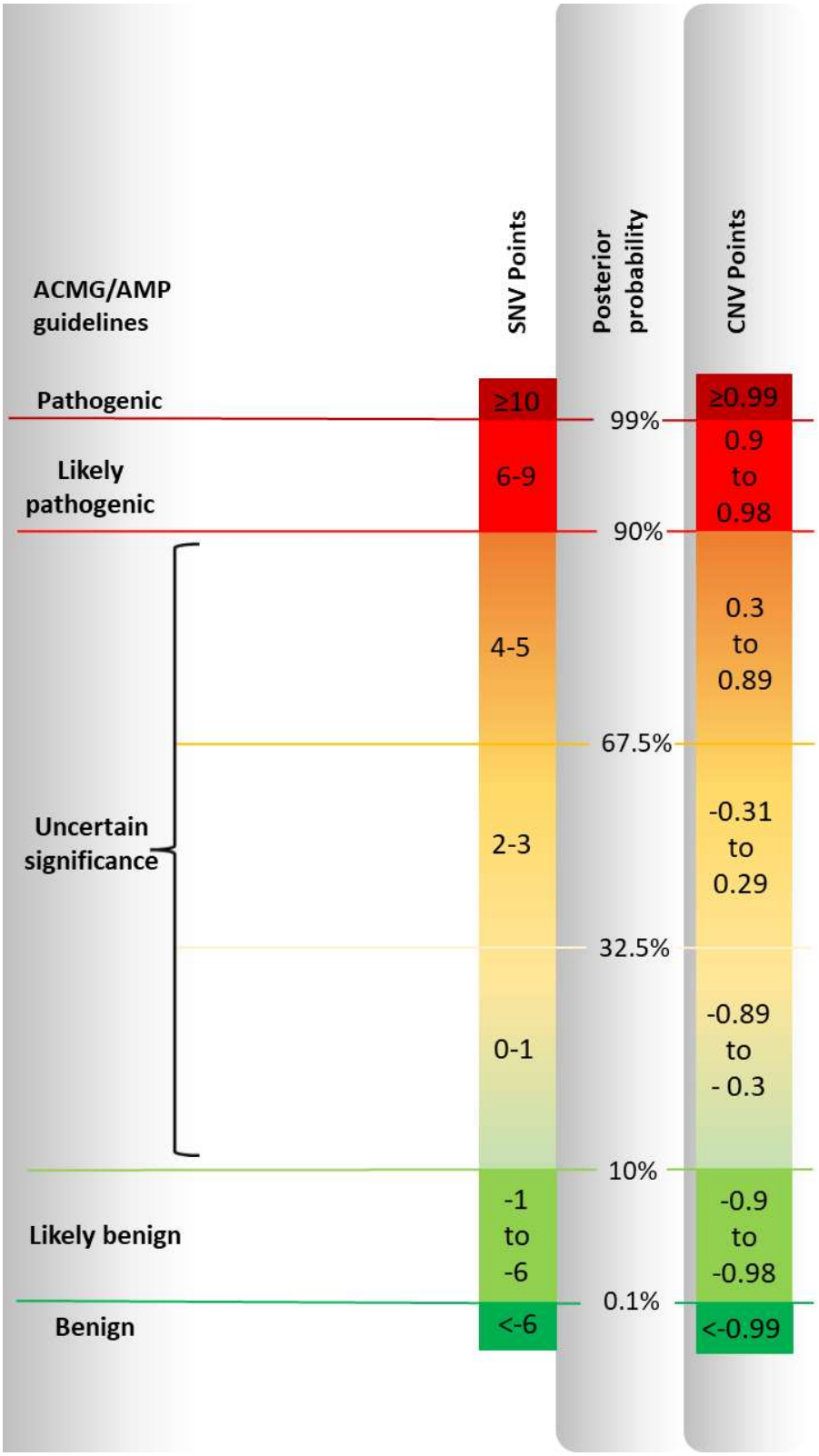
### 7.1 Variants of uncertain significance

The reporting of variants of uncertain significance can be challenging and the consequences of a misdiagnosis due to misunderstanding the significance of a reported variant of uncertain significance may have wider implications beyond the proband. It is essential to use clinical judgement and consider discussion in a multidisciplinary setting (i.e. with the referring clinician).

With the caveat that current variant classifications are not quantitative, the likelihood of a variant of uncertain significance being pathogenic is intended to range from 10% to 90% (Richards *et al*., 2015). They also noted that *"some laboratories may choose to sub-classify VUSs, particularly for internal use"*. **Figure 3** illustrates sub-classification into three VUS groups, using posterior probabilities estimated from a Bayesian approach and on a points scale (derived from the Bayesian approach). The aim of **Figure 3** is to convey the different levels of uncertainty within the "variant of uncertain significance" category. Whilst laboratories and clinicians may find it helpful to use VUS sub-classifications, these should not be included in the genomic laboratory report.

**Evidence points for pathogenic (P) codes**: Very Strong= 8, Strong= 4, Moderate= 2, Supporting= 1.
**Evidence points for benign (B) codes:** Strong= -4, Moderate= -2, Supporting= -1.

**Figure 3: The point systems used to classify variants using SNV and CNV guidelines with differing levels of evidence in support of pathogenicity**



### 7.1.1. Situations where considering reporting a VUS might be appropriate

**Variants of uncertain significance should generally only be considered for reporting where there is a high level of supporting evidence and additional evidence might be obtained to allow re-classification as (likely) pathogenic.**

This might include discussion in a multidisciplinary setting where possible (i.e. with the referring clinician) to determine whether parental samples might be available to demonstrate a *de novo* variant (PS2/PM6 - SNV guidelines, 5A - CNV guidelines) and parental relationships, if there are sufficient affected relatives (number of informative meiosis) available to show co-segregation (PP1 – SNV guidelines, 5D - CNV guidelines), whether neuroimaging/muscle biopsy or a biochemical test could provide phenotype specificity evidence (PP4 – SNV guidelines, Section 5 - CNV guidelines), whether trial of a treatment that is specific for the genetic aetiology (e.g. biotin in a patient with biallelic *BTH* variants) or mRNA analysis in support of aberrant splicing (PVS1(RNA) – SNV guidelines). If additional evidence could allow re-classification of the variant as (likely) pathogenic, the initial report should clearly state the further action to be considered and explain how this might change the variant classification to likely pathogenic. We recommend that the following text is included in the Result Summary box: "Variant of uncertain significance identified – consider further action" and that the further investigations or tests that might be undertaken are clearly detailed in the "Recommended action" section.

Please note that the emphasis is on additional testing to obtain evidence in support of pathogenicity. In some cases, the new information will re-classify the variant of uncertain significance as likely benign, but routine practice should not include additional testing to prove that a variant with little supporting evidence in favour of pathogenicity is benign. When a VUS is reported it should be made clear that this finding in isolation is insufficient to justify a change in clinical management.

**<u>Exceptional circumstances</u>**

There may be situations in which an MDT discussion concludes that there is clinical utility in reporting a variant of uncertain significance. Usually this would be a situation where it may be impossible to obtain sufficient evidence at this time to reach a variant classification of (likely) pathogenic, but where all the available clinical, gene-level and variant-level evidence supports the likely diagnosis. One scenario might be a rare autosomal recessive disease with a specific phenotype and one pathogenic or likely pathogenic variant plus a VUS with a high level of supporting evidence. In this situation we recommend that the following text is included in the Result Summary box: "Possible genetic diagnosis of disorder X – additional evidence required to clarify this result" and the additional evidence required is stated in the report.

Where there is moderate evidence for pathogenicity, the prior probability of pathogenicity is particularly important. For example, if there are multiple affected individuals within the family, the specific gene/region (or biological pathway) is indicated by the referring clinician or the clinical presentation suggests a very high likelihood of the genetic disorder.

In most clinical settings and for most genes/regions, variants of uncertain significance with a low level of supportive evidence are almost invariably unlikely to be disease-causing and should only be reported in exceptional circumstances. MDT discussion may be helpful for determining

this. These are often novel variants (absent from literature and databases). Most frequently they are either missense variants in genes with missense constraint (PP2) or for which *in silico* tools predict a deleterious effect on protein function (PP3). This level of evidence should be considered circumstantial evidence in the absence of a high level of phenotypic specificity. Novel contiguous gene CNVs that do not contain any established disease-associated genes linked to the patient's phenotype or have the required number of genes to apply 3C, but often contain many predicted dosage sensitive genes, is another example. In the absence of any literature or database evidence, determining if these CNVs have arisen *de novo* or are segregating with a phenotype within a family may not result in enough additional points to change the classification of these variants, especially when detected in patients with non-specific phenotypes. It is recommended that these CNVs and phenotypes are added to DECIPHER to help with the future interpretation of these regions.

There are some specific exceptional circumstances where a prior decision may be made by the laboratory and expert clinical team to report certain variants of uncertain significance in specific genes. Examples include where a well-established specific pharmacological therapy is recommended for a genetic disorder and a treatment trial may be considered for certain variants of uncertain significance. For example, low dose sulphonylurea therapy is recommended for patients with (likely) pathogenic *HNF1A* or *HNF4A* variants causing monogenic diabetes (Pearson *et al*., 2003) and biotin treatment is effective for patients with biallelic (likely) pathogenic *BTH* variants.

#### 7.1.2. Situations where reporting a VUS would not be considered appropriate

**There are some additional situations where VUSs should not be reported. These include:**

(i) Variants reported in the published literature and mutation databases as pathogenic/likely pathogenic for which subsequent scientific evidence has re-classified the variant as likely benign or benign. Examples include the *RET* p.(Tyr791Phe) missense variant (Toledo *et al*., 2015) and *BRCA1* c.594-2A>C (de la Hoya *et al*., 2016). There is no clinical utility in reporting these historical false positive results and past experience has demonstrated the potential for risk of misinterpretation of such information.

(ii) Variant type/mechanism that does not fit with the established disease mechanism, for example protein truncating variants predicted to result in nonsense-mediated decay in a gene where the known disease mechanism is gain of function due to activating missense variants, or loss of function variants in *NOTCH3* in CADASIL, where this variant type is well established to be benign.

(iii) Heterozygous VUS in a gene only associated with an autosomal recessive disease, where a second candidate variant has not been detected.

(iv) CNV with a negative points score.

### 7.1.3. Specific VUS reporting considerations related to WGS, WES and large gene panel tests

An evidence-based framework for assessing gene-disease associations has been developed by the ClinGen group (Strande *et al*., 2017). Variants identified within a *"gene of uncertain significance"* should always be classified as variants of uncertain significance (Richards *et al*., 2015). Such variants should only be included in the genomic laboratory report if there is robust evidence for the gene-disease association and publication is pending. An updated report should be issued after publication to include the reference.

The advent of genome sequencing has not only identified many new disease genes not previously associated with human disease, but also identified novel phenotypes linked to known disease genes, thereby expanding the phenotypic spectrum. For the purposes of clinical diagnostic testing, the focus must be on known gene-disease associations. For example, if a novel missense variant is identified in a gene associated with a particular set of phenotypic features but the patient's clinical presentation fits with only a single/subset of non-specific features of the disorders or is out-with the phenotypic spectrum associated with the disease gene, we might hypothesise that the restricted clinical presentation is a consequence of the missense variant being hypomorphic or that the patient represents an extension of the currently established disease spectrum. In the absence of other phenotypically-matched patients with hypomorphic variants confirmed by functional studies, a case series establishing a new phenotype association with the disease gene or additional robust evidence, such variants should not be reported.

## 7.2 Reporting a heterozygous (likely) pathogenic variant for a gene associated with an autosomal recessive disease

For single gene or targeted testing where the prior probability of a particular autosomal recessive disorder is high, the finding of a single monoallelic variant involving a gene associated with the autosomal recessive disorder would be reported as "at least a carrier and this result increases the likelihood of a diagnosis of disorder X". An example would be a patient with a positive sweat test undergoing testing for common *CFTR* variants. This reporting rationale is based on (a) the prior probability from the phenotype and (b) the incomplete nature of the test i.e. testing only the most common pathogenic *CFTR* variants.

When testing large gene panels or undertaking a chromosomal microarray, if a single monoallelic (likely) pathogenic variant involving a gene associated with an autosomal recessive disorder is found it means that either (a) incidental carrier status has been revealed or (b) a second variant has been inherited *in trans* but has not been detected. If the patient's phenotype is not compatible with the disorder or (biallelic) variant(s) explaining the phenotype have been identified in another gene, then this is likely to be an incidental finding.

Reporting of carrier status in these scenarios as a default approach is ***not recommended*** because such variants are not of relevance to the clinical presentation. Every individual is likely to be a carrier for multiple rare diseases and reporting those variants in the context of a test to investigate the cause of the patient's rare disorder has greater potential to mislead than to appropriately inform.

In the situation that we find a single monoallelic (likely) pathogenic variant in a gene associated with an autosomal recessive disorder and the patient's phenotype is compatible with this disorder, then the decision to report will depend upon the phenotypic specificity, size of the gene

panel, known clinical sensitivity of the test and whether a second variant might be detected by additional analysis of the genomic data or another diagnostic testing method. **Therefore, single heterozygous variants in autosomal recessive genes should not be reported unless there is additional testing that the laboratory would recommend that is likely to help confirm the diagnosis in the proband**.

The NHS England Genomics Unit "Guidelines for Rare Disease Whole Genome Sequencing & Next Generation Sequencing Panel Interpretation & Reporting"" has further guidance for healthcare scientists on when and how to report incidental/unexpected findings https://future.nhs.uk/NHSgenomics/view?objectId=154355013.

### 7.3 Reporting of reduced penetrance or hypomorphic variants

Where lower penetrance genes or genetic variants are included in a gene panel test, any lower penetrance pathogenic variant(s) identified are unlikely to account for the majority of the phenotype/risk and this should be clearly articulated.

Reduced penetrance and hypomorphic variants should only be reported in relevant clinical contexts. These variants, especially those with limited evidence, can be difficult to classify using current guidelines and should be clearly labelled in reports.

#### 7.3.1 Reduced penetrance variants

Reduced penetrance variants cause disease with the same inheritance pattern as fully penetrant variants, however, may show later age-of-onset, different spectrum of clinical features and/or be asymptomatic. Many recurrent microduplication/microdeletion CNVs are associated with incomplete penetrance and variable expressivity. Some CNV regions are more highly penetrant than others (Rosenfeld *et al*., 2013; Kendall *et al*., 2019). The recommended terminology is e.g. "(likely) pathogenic, reduced penetrance"; "(likely) pathogenic, low penetrance", "(likely) pathogenic with low penetrance and variable expressivity" etc (Schmidt *et al.,* 2023).

Example report wording for report Result Summary: "Consistent with a genetic diagnosis of disorder X with reduced penetrance OR Consistent/associated with a genetic susceptibility to disorder X"

> Example 1: The most frequently reported *HNF4A* pathogenic variant p.(Arg114Trp) has high frequency in population studies, lack of co-segregation in some pedigrees and inconsistent functional studies. A case-control study confirmed it is a pathogenic variant causing a distinct clinical subtype of *HNF4A* MODY with reduced penetrance, reduced sensitivity to sulfonylurea treatment and no effect on birth weight (Laver *et al*., 2016).
>
> Example 2: Penetrance of the *CFTR* variant p.(Arg117His) is variable and affected by the length of the intron 8 polyT tract. When inherited *in trans* with a severe *CFTR* pathogenic variant such as p.(Phe508del), p.(Arg117His) with 5T is likely to be associated with variable clinical symptoms of cystic fibrosis (CF) with pancreatic sufficiency; p.(Arg117His) with 7T is unlikely to cause classic CF and may be associated with male infertility and/or mild clinical symptoms of CF; p.(Arg117His) with 9T is highly unlikely to act as a disease-causing variant and is not associated with male infertility.
>
> Example 3: Heterozygous deletion of the recurrent proximal 16p11.2 region (BP4-BP5) is pathogenic. The phenotype is associated with low penetrance and variable expressivity; these deletions have been observed in individuals with neurodevelopmental disorders as well as in apparently normal individuals,

even within the same family. This deletion is likely to be contributing to this patient's clinical phenotype. Clinical correlation is recommended to determine if additional testing is warranted.

The reporting of recurrent microduplication/microdeletion CNVs associated with incomplete penetrance and variable expressivity is dependent on the clinical presentation of the patient and reason for testing. Irrespective of their classification, it is not recommended that these CNVs are reported in the absence of a neurodevelopmental phenotype and therefore should be treated as incidental/unexpected findings in such clinical scenarios.

### 7.3.2 Hypomorphic variants

Hypomorphic variants show a partial loss of gene function that may only result in a clinical phenotype when inherited with another deleterious variant and may show a different inheritance pattern or phenotype to full or reduced penetrance variants. Such variants should only be reported in appropriate clinical contexts.

The main body of the report should clearly state that the variant has been shown to be hypomorphic and is associated with a specific phenotype/inheritance pattern.

Example 1: Polycystic kidney disease due to heterozygous *PKD1* pathogenic variants is usually an adult onset, autosomal dominant disorder (ADPKD). However, rare, severe, very early onset (VEO) PKD is caused by biallelic *PKD1* variants, including at least one hypomorphic variant. Biallelic *PKD1* pathogenic variants are embryonic lethal. The c.9829C>T p.(Arg3277Cys) variant is the most widely reported and characterised hypomorphic *PKD1* variant. There is sufficient evidence, including functional studies, to classify this variant as pathogenic (or an established hypomorphic variant) in VEO-PKD. However, it is frequent in gnomAD and does not cause cysts in isolation in heterozygotes; therefore, in heterozygous state in classic adult-onset ADPKD patients, it should be classified as a VUS/established hypomorphic variant and should not be reported. Homozygotes for this variant do develop adult-onset PKD including end stage renal disease therefore it is appropriate to report homozygotes (Rossetti *et al*., 2008). A second common hypomorphic variant c.9499A>T p.(Ile3167Phe) causing VEO-PKD has recently been reported with high gnomAD frequency (>0.2%) therefore care is required to ensure relevant hypomorphic variants are not automatically filtered for such cases (Durkie *et al*., 2023).

Example 2: Autosomal recessive fumarate hydratase deficiency (FHD) is a severe, neonatal or early infantile metabolic encephalopathy disorder, associated with biallelic variants in the *FH* gene. Monoallelic pathogenic variants in the same gene are associated with autosomal dominant Hereditary Leiomyomas & Renal Cell Cancer (HLRCC). Biallelic pathogenic *FH* variants are assumed to be embryonic lethal; therefore, one or both of the variants detected in FHD cases are likely hypomorphic and care should be taken when reporting in the context of HLRCC. The most common pathogenic variant seen in FHD is c.1431_1433dup p.(Lys477dup) however, it is present in 0.1% of population in gnomAD and there is no evidence of an association with HLRCC, even amongst the parents of FHD patients (Zhang *et al*., 2020); therefore, it should not be reported in HLRCC referrals.

Example 3: Autosomal recessive Stargardt disease is caused by variants in the *ABCA4* gene. The deep intronic c.4253+43G>A variant is found in 0.6% of non-Finnish European alleles and in ~3% of STGD1 patients. The variant, which is not predicted to have any effect on splicing, is the first reported intronic "extremely hypomorphic allele" in the *ABCA4* locus and is pathogenic only when *in trans* with a loss-of-function *ABCA4* allele (Zernant *et al*., 2018).

### 7.4 Reporting of risk (susceptibility) alleles

Well established SNV variants that confer a small increased risk of disease (odds ratio (OR) >2) may be reported, in appropriate clinical contexts, as an established/likely/uncertain risk allele, based on recommendations from ClinGen low penetrance / risk allele working group (Schmidt *et al.,* 2023). The framework to determine their classification is based on the strength of association studies (very strong/strong/limited evidence) depending on the number of cases vs controls ± functional studies.

Example report wording for report Result Summary: "Consistent/associated with a genetic susceptibility to disorder X".

Example wording for main report: "This established risk allele is common in population studies; however, it is associated with an increased risk of disorder X <add OR if available>, therefore it is likely consistent/associated with their clinical presentation".

Examples of established risk alleles with very strong association studies (meta-analysis) include: *HFE:* c.845G>A p.(Cys282Tyr) common variant associated with Hereditary Haemochromatosis; 75–85% of homozygotes do not develop HFE-related HH (Mayr *et al*., 2010)); *SPINK1:* c.101A>G p.(Asn34Ser) common variant (~1% of NFE alleles on gnomAD) in linkage disequilibrium with promoter variant c.-192+3416G>T is associated with increased risk of chronic pancreatitis with odds ratio 6.82 for heterozygotes and odds ratio 97.74 for homozygotes (Di Leo *et al*., 2017).

Some recurrent microduplication/microdeletion CNVs are considered susceptibility/risk loci for neurodevelopmental disorders e.g. 15q11.2 recurrent microdeletion (BP1-BP2; includes *NIPA1*), 16p12.2 recurrent microdeletion (proximal; includes *EEF2K, CDR2*), 16p13.11 recurrent microduplication/microdeletion (BP2-BP3; includes *MYH11*). Irrespective of their classification, it is not recommended that these CNVs are reported in the absence of a neurodevelopmental phenotype and therefore should be treated as incidental/unexpected findings in such clinical scenarios.

## 8. Storage of variant data for future re-analysis

Next generation sequencing of large gene panels, a whole exome or whole genome will identify multiple variants of uncertain significance. Some pathogenic variants may not be detected using standard bioinformatics filtering e.g. lie within poorly sequenced regions, gene not yet known to be associated with the disease phenotype, types of variants not detectable by the bioinformatics pipeline, variants within regulatory elements, deep intronic cryptic splicing variants or mosaic variants. Therefore, it is important to store sequence data in an accessible, secure way to permit future re-analysis within a clinically appropriate timeframe within the laboratory. A systematic process for retrieving an individual patient's data is essential.

## 9. Reclassification of variants

Variant data and relevant associated information must be stored within the laboratory in a way that allows reclassification if required. We ***recommend*** that the evidence supporting the variant classification is included in an appendix to the report for reportable VUS and likely pathogenic variants. National guidance is available and defines criteria for re-analysis of stored genomic data for patients in whom no analysis is found during the initial testing process.

Reassessment of a variant that results in reclassification may be prompted by the publication of new knowledge regarding the variant (or genotype-phenotype association); by a request for a

family member test or as a result of further clinical investigations or evolution of the patient's phenotype that questions the original diagnosis.

Reclassification of a variant across categories that fundamentally changes the clinical relevance – i.e. not from likely benign to benign or likely pathogenic to pathogenic (or vice versa) – should be shared with other relevant health care professionals. The laboratory where the new information is generated should liaise with any laboratories (where feasible) that generated the original classification to ensure consistency across centres. The new classification data and the basis for this classification should be placed in a publicly accessible database e.g. ClinVar, so that the information is available widely. If the new classification has potential importance for clinical management e.g. classification of a *BRCA1* or *BRCA2* variant that may alter decisions around risk-reducing surgery, this decision should be documented (through an MDT) and communicated to the patient's clinical team(s) as quickly as possible. For some variants, for example the re-classification of *BRCA1* c.594-2A>C (de la Hoya *et al*., 2016), rapid communication of this information to all other UK diagnostic genetics laboratories is clearly appropriate (using a designated secure NHS email address for each laboratory). An urgent email alert for all NHS registered users of https://canvaruk.org/ is available to alert users to clinically relevant changes in variant classification in cancer susceptibility genes.

Variant classification guidelines are evolving rapidly with dedicated ClinGen groups focussing on specific evidence codes, disease-specific VCEPs and an ongoing overhaul of the ACMG framework to a points-based system. Therefore, variants may also be reclassified due to a change in guidelines, with no change in the preceding evidence for pathogenicity. In cases where the reclassification changes from borderline likely pathogenic to borderline VUS, caution should be exercised as this classification is potentially changeable as guidelines evolve. Recommendations for laboratory reporting, including report wording and clinical management in such cases for cancer susceptibility genes is available in (Loong *et al*., 2022) and may also be applicable for other rare disease cases.

It is the professional responsibility of the Clinical Genomic community to ensure data is shared responsibly for improved patient care, using the CanVar-UK database for cancer predisposition gene variants and ClinVar for all rare disease variants.

**Acknowledgements**

We are very grateful to Dr Steven Harrison and Professor Heidi Rehm for their expert leadership, collaboration and support that underpins these guidelines. We thank the co-authors of the previous versions (Alison Callaway, Natalie Forrester, Diana Eccles, Stephen Abbs, Tracy Lester, Jo Campbell, William Newman and Simon Ramsden), and all our colleagues from the Association for Clinical Genomic Science who have contributed to these guidelines.

**References**

Abou Tayoun, A. N., Pesaran, T., DiStefano, M. T., Oza, A., Rehm, H. L., Biesecker, L. G., & Harrison, S. M. (2018). Recommendations for interpreting the loss of function PVS1 ACMG/AMP variant criterion. *Human Mutation*, *39*(11), 1517–1524. https://doi.org/10.1002/HUMU.23626

Brnich, S. E., Abou Tayoun, A. N., Couch, F. J., Cutting, G. R., Greenblatt, M. S., Heinen, C. D., Kanavy, D. M., Luo, X., McNulty, S. M., Starita, L. M., Tavtigian, S. V., Wright, M. W., Harrison, S. M., Biesecker, L. G., Berg, J. S., Abou Tayoun, A. N., Berg, J. S., Biesecker, L. G., Brenner, S. E., … Topper, S. (2019). Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework. In *Genome Medicine* (Vol. 12, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13073-019-0690-2

Cannon, S., Williams, M., Gunning, A. C., & Wright, C. F. (2023). Evaluation of in silico pathogenicity prediction tools for the classification of small in-frame indels. *BMC Medical Genomics*, *16*(1). https://doi.org/10.1186/s12920-023-01454-6

Cheng, J., Novati, G., Pan, J., Bycroft, C., Žemgulytė, A., Applebaum, T., Pritzel, A., Wong, L. H., Zielinski, M., Sargeant, T., Schneider, R. G., Senior, A. W., Jumper, J., Hassabis, D., Kohli, P., & Avsec, Ž. (2023). Accurate proteome-wide missense variant effect prediction with AlphaMissense. *Science*, 381. https://doi.org/10.1126/science.adg7492

Cubuk, C., Garrett, A., Choi, S., King, L., Loveday, C., Torr, B., Burghel, G. J., Durkie, M., Callaway, A., Robinson, R., Drummond, J., Berry, I., Wallace, A., Eccles, D., Tischkowitz, M., Whiffin, N., Ware, J. S., Hanson, H., Turnbull, C., & CanVIG-UK. (2021). Clinical likelihood ratios and balanced accuracy for 44 in silico tools against multiple large-scale functional assays of cancer susceptibility genes. *Genetics in Medicine: 23*(11), 2096–2104. https://doi.org/10.1038/S41436-021-01265-Z

de la Hoya, M., Soukarieh, O., López-Perolio, I., Vega, A., Walker, L. C., van Ierland, Y., Baralle, D., Santamariña, M., Lattimore, V., Wijnen, J., Whiley, P., Blanco, A., Raponi, M., Hauke, J., Wappenschmidt, B., Becker, A., Hansen, T. V. O., Behar, R., KConFaB Investigators, … Spurdle, A. B. (2016). Combined genetic and splicing analysis of BRCA1 c.[594-2A>C; 641A>G] highlights the relevance of naturally occurring in-frame transcripts for developing disease gene variant classification algorithms. *Human Molecular Genetics*, *25*(11), 2256–2268. https://doi.org/10.1093/HMG/DDW094

Di Leo, M., Bianco, M., Zuppardo, R. A., Guslandi, M., Calabrese, F., Mannucci, A., Neri, T. M., Testoni, P. A., Leandro, G., & Cavestro, G. M. (2017). Meta-analysis of the impact of SPINK1 p.N34S gene variation in Caucasic patients with chronic pancreatitis. An update. *Digestive and Liver Disease, 49*(8), 847–853. https://doi.org/10.1016/J.DLD.2017.04.023

Durkie, M., Watson, C. M., Winship, P., Hogg, A.-C., Nyanhete, R., Cooley, S., Valluru, M. K., Shaw-Smith, C., Bingham, C., Gilchrist, M., Kenny, J., Consortium, G. E. R., & Ong, A. C. M. (2023). The Common PKD1 p.(Ile3167Phe) Variant Is Hypomorphic and Associated with Very Early Onset, Biallelic Polycystic Kidney Disease. *Human Mutation*, 2023, 1–8. https://doi.org/10.1155/2023/5597005

Ellingford, J. M., Ahn, J. W., Bagnall, R. D., Baralle, D., Barton, S., Campbell, C., Downes, K., Ellard, S., Duff-Farrier, C., FitzPatrick, D. R., Greally, J. M., Ingles, J., Krishnan, N., Lord, J., Martin, H. C., Newman, W. G., O'Donnell-Luria, A., Ramsden, S. C., Rehm, H. L., … Whiffin, N. (2022). Recommendations for clinical interpretation of variants found in non-coding regions of the genome. *Genome Medicine*, *14*(1). https://doi.org/10.1186/s13073-022-01073-3

Feng, B. J. (2017). PERCH: A Unified Framework for Disease Gene Prioritization. *Human Mutation*, *38*(3), 243–251. https://doi.org/10.1002/HUMU.23158

Garrett, A., Callaway, A., Durkie, M., Cubuk, C., Alikian, M., Burghel, G. J., Robinson, R., Izatt, L., Talukdar, S., Side, L., Cranston, T., Palmer-Smith, S., Baralle, Di., Berry, I. R., Drummond, J., Wallace, A. J., Norbury, G., Eccles, Di. M., Ellard, S., … Turnbull, C. (2020). Cancer Variant Interpretation Group UK (CanVIG-UK): An exemplar national subspecialty multidisciplinary network. *Journal of Medical Genetics*, *57*(12), 829–834. https://doi.org/10.1136/jmedgenet-2019-106759

Garrett, A., Durkie, M., Callaway, A., Burghel, G. J., Robinson, R., Drummond, J., Torr, B., Cubuk, C., Berry, I. R., Wallace, A. J., Ellard, S., Eccles, D. M., Tischkowitz, M., Hanson, H., & Turnbull, C. (2021). Combining evidence for and against pathogenicity for variants in cancer susceptibility genes: CanVIG-UK consensus recommendations. *Journal of Medical Genetics, 58*(5). https://doi.org/10.1136/jmedgenet-2020-107248

Goldberg, Y., Laitman, Y., Ben David, M., Bazak, L., Lidzbarsky, G., Salmon, L. B., Shkedi-Rafid, S., Barshack, I., Avivi, C., Darawshe, M., Shomron, N., Bruchim, R., Vinkler, C., Yannoukakos, D., Fostira, F., Bernstein-Molho, R., & Friedman, E. (2021). Re-evaluating the pathogenicity of the c.783+2T>C BAP1 germline variant. *Human Mutation*, *42*(5), 592–599. https://doi.org/10.1002/HUMU.24189

Havrilla, J. M., Pedersen, B. S., Layer, R. M., & Quinlan, A. R. (2018). A map of constrained coding regions in the human genome. *Nature Genetics, 51:1*, *51*(1), 88–95. https://doi.org/10.1038/s41588-018-0294-6

Ioannidis, N. M., Rothstein, J. H., Pejaver, V., Middha, S., McDonnell, S. K., Baheti, S., Musolf, A., Li, Q., Holzinger, E., Karyadi, D., Cannon-Albright, L. A., Teerlink, C. C., Stanford, J. L., Isaacs, W. B., Xu, J., Cooney, K. A., Lange, E. M., Schleutker, J., Carpten, J. D., … Sieh, W. (2016). REVEL: An Ensemble Method for Predicting the Pathogenicity of Rare Missense Variants. *American Journal of Human Genetics*, *99*(4), 877–885. https://doi.org/10.1016/j.ajhg.2016.08.016

Jarvik, G. P., & Browning, B. L. (2016). Consideration of Cosegregation in the Pathogenicity Classification of Genomic Variants. *American Journal of Human Genetics, 98*(6), 1077–1081. https://doi.org/10.1016/j.ajhg.2016.04.003

Kelly, M. A., Caleshu, C., Morales, A., Buchan, J., Wolf, Z., Harrison, S. M., Cook, S., Dillon, M. W., Garcia, J., Haverfield, E., Jongbloed, J. D. H., Macaya, D., Manrai, A., Orland, K., Richard, G., Spoonamore, K., Thomas, M., Thomson, K., Vincent, L. M., … Wilde, A. (2018). Adaptation and validation of the ACMG/AMP variant classification framework for MYH7-associated inherited cardiomyopathies: recommendations by ClinGen's Inherited Cardiomyopathy Expert Panel. *Genetics in Medicine: 20*(3), 351–359. https://doi.org/10.1038/GIM.2017.218

Kendall, K. M., Bracher-Smith, M., Fitzpatrick, H., Lynham, A., Rees, E., Escott-Price, V., Owen, M. J., O'Donovan, M. C., Walters, J. T. R., & Kirov, G. (2019). Cognitive performance and functional outcomes of carriers of pathogenic copy number variants: analysis of the UK Biobank. *The British Journal of Psychiatry : The Journal of Mental Science*, *214*(5), 297–304. https://doi.org/10.1192/bjp.2018.301

Laver, T. W., Colclough, K., Shepherd, M., Patel, K., Houghton, J. A. L., Dusatkova, P., Pruhova, S., Morris, A. D., Palmer, C. N., McCarthy, M. I., Ellard, S., Hattersley, A. T., & Weedon, M. N. (2016). The Common p.R114W HNF4A Mutation Causes a Distinct Clinical Subtype of Monogenic Diabetes. *Diabetes*, *65*(10), 3212–3217. https://doi.org/10.2337/DB16-0628

Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., O'Donnell-Luria, A. H., Ware, J. S., Hill, A. J., Cummings, B. B., Tukiainen, T., Birnbaum, D. P., Kosmicki, J. A., Duncan, L. E., Estrada, K., Zhao, F., Zou, J., Pierce-Hoffman, E., Berghout, J., … Williams, A. L. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature*, *536*(7616), 285–291. https://doi.org/10.1038/NATURE19057

Lin, J. H., Masson, E., Boulling, A., Hayden, M., Cooper, D. N., Férec, C., Liao, Z., & Chen, J. M. (2020). 5' splice site GC>GT and GT>GC variants differ markedly in terms of their functionality and pathogenicity. *Human Mutation, 41*(8), 1358–1364. https://doi.org/10.1002/HUMU.24029

Lindeboom, R. G. H., Supek, F., & Lehner, B. (2016). The rules and impact of nonsense-mediated mRNA decay in human cancers. *Nature Genetics*, *48*(10), 1112–1118. https://doi.org/10.1038/ng.3664

Loong, L., Garrett, A., Allen, S., Choi, S., Durkie, M., Callaway, A., Drummond, J., Burghel, G. J., Robinson, R., Torr, B., Berry, I. R., Wallace, A. J., Eccles, D. M., Ellard, S., Baple, E., Evans, D. G., Woodward, E. R., Kulkarni, A., Lalloo, F., … Turnbull, C. (2022). Reclassification of clinically-detected sequence variants: Framework for genetic clinicians and clinical scientists by CanVIG-UK (Cancer Variant Interpretation Group UK). *Genetics in Medicine*, *24*(9), 1867–1877. https://doi.org/10.1016/j.gim.2022.05.002

Lord, J., Gallone, G., Short, P. J., McRae, J. F., Ironfield, H., Wynn, E. H., Gerety, S. S., He, L., Kerr, B., Johnson, D. S., McCann, E., Kinning, E., Flinter, F., Temple, I. K., Clayton-Smith, J., McEntagart, M., Lynch, S. A., Joss, S., Douzgou, S., … Deciphering Developmental Disorders study. (2019). Pathogenicity and selective constraint on variation near splice sites. *Genome Research*, *29*(2), 159–170. https://doi.org/10.1101/gr.238444.118

Mavraki, E., Labrum, R., Sergeant, K., Alston, C. L., Woodward, C., Smith, C., Knowles, C. V. Y., Patel, Y., Hodsdon, P., Baines, J. P., Blakely, E. L., Polke, J., Taylor, R. W., & Fratter, C. (2023). Genetic testing for mitochondrial disease: the United Kingdom best practice guidelines. *European Journal of Human Genetics, 31*(2), 148–163. https://doi.org/10.1038/s41431-022-01249-w

Mayr, R., Janecke, A. R., Schranz, M., Griffiths, W. J. H., Vogel, W., Pietrangelo, A., & Zoller, H. (2010). Ferroportin disease: a systematic meta-analysis of clinical and molecular findings. *Journal of Hepatology*, *53*(5), 941–949. https://doi.org/10.1016/J.JHEP.2010.05.016

Mazoyer, S., Dunning, A. M., Serova, O., Dearden, J., Puget, N., Healey, C. S., Gayther, S. A., Mangion, J., Stratton, M. R., Lynch, H. T., Goldgar, D. E., Ponder, B. A. J., & Lenoir, G. M. (1996). A polymorphic stop codon in BRCA2. *Nature Genetics*, *14*(3), 253–254. https://doi.org/10.1038/NG1196-253

McCormick, E. M., Lott, M. T., Dulik, M. C., Shen, L., Attimonelli, M., Vitale, O., Karaa, A., Bai, R., Pineda-Alvarez, D. E., Singh, L. N., Stanley, C. M., Wong, S., Bhardwaj, A., Merkurjev, D., Mao, R., Sondheimer, N., Zhang, S., Procaccio, V., Wallace, D. C., … Falk, M. J. (2020). Specifications of the ACMG/AMP standards and guidelines for mitochondrial DNA variant interpretation. *Human Mutation*, *41*(12), 2028–2057. https://doi.org/10.1002/HUMU.24107

Newman, S., Hermetz, K. E., Weckselblatt, B., & Rudd, M. K. (2015). Next-generation sequencing of duplication CNVs reveals that most are tandem and some create fusion genes at breakpoints. *American Journal of Human Genetics*, *96*(2), 208–220. https://doi.org/10.1016/J.AJHG.2014.12.017

Nix, P., Mundt, E., Coffee, B., Goossen, E., Warf, B. M., Brown, K., Bowles, K., & Roa, B. (2022). Interpretation of BRCA2 Splicing Variants: A Case Series of Challenging Variant Interpretations and the Importance of Functional RNA Analysis. *Familial Cancer*, *21*(1), 7–19. https://doi.org/10.1007/S10689-020-00224-Y

Pearson, E. R., Starkey, B. J., Powell, R. J., Gribble, F. M., Clark, P. M., & Hattersley, A. T. (2003). Genetic cause of hyperglycaemia and response to treatment in diabetes. *Lancet (London, England)*, *362*(9392), 1275–1281. https://doi.org/10.1016/S0140-6736(03)14571-0

Pejaver, V., Byrne, A. B., Feng, B. J., Pagel, K. A., Mooney, S. D., Karchin, R., O'Donnell-Luria, A., Harrison, S. M., Tavtigian, S. V., Greenblatt, M. S., Biesecker, L. G., Radivojac, P., Brenner, S. E., Tayoun, A. A., Berg, J. S., Cutting, G. R., Ellard, S., Kang, P., Karbassi, I., … Topper, S. (2022). Calibration of computational tools for missense variant pathogenicity classification and ClinGen recommendations for PP3/BP4 criteria. *American Journal of Human Genetics*, *109*(12), 2163–2177. https://doi.org/10.1016/j.ajhg.2022.10.013

Posey, J. E., Harel, T., Liu, P., Rosenfeld, J. A., James, R. A., Coban Akdemir, Z. H., Walkiewicz, M., Bi, W., Xiao, R., Ding, Y., Xia, F., Beaudet, A. L., Muzny, D. M., Gibbs, R. A., Boerwinkle, E., Eng, C.

M., Sutton, V. R., Shaw, C. A., Plon, S. E., … Lupski, J. R. (2017). Resolution of Disease Phenotypes Resulting from Multilocus Genomic Variation. *The New England Journal of Medicine*, *376*(1), 21–31. https://doi.org/10.1056/NEJMOA1516767

Richards, S., Aziz, N., Bale, S., Bick, D., Das, S., Gastier-Foster, J., Grody, W. W., Hegde, M., Lyon, E., Spector, E., Voelkerding, K., & Rehm, H. L. (2015). Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine*, *17*(5), 405–424. https://doi.org/10.1038/gim.2015.30

Riggs, E. R., Andersen, E. F., Cherry, A. M., Kantarci, S., Kearney, H., Patel, A., Raca, G., Ritter, D. I., South, S. T., Thorland, E. C., Pineda-Alvarez, D., Aradhya, S., Martin, C. L., & on behalf of the ACMG. (2020). Technical standards for the interpretation and reporting of constitutional copy-number variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics (ACMG) and the Clinical Genome Resource (ClinGen). *Genetics in Medicine, 22*(2), 245–257. https://doi.org/10.1038/S41436-019-0686-8

Rosenfeld, J. A., Coe, B. P., Eichler, E. E., Cuckle, H., & Shaffer, L. G. (2013). Estimates of penetrance for recurrent pathogenic copy-number variations. *Genetics in Medicine, 15*(6), 478–481. https://doi.org/10.1038/gim.2012.164

Rossetti, S., Kubly, V. J., Consugar, M. B., Hopp, K., Roy, S., Horsley, S. W., Chauveau, D., Rees, L., Barratt, T. M., Van't Hoff, W. G., Niaudet, W. P., Torres, V. E., & Harris, P. C. (2009). Incompletely penetrant PKD1 alleles suggest a role for gene dosage in cyst initiation in polycystic kidney disease. *Kidney International, 75*(8), 848–855. https://doi.org/10.1038/ki.2008.686

Rowlands, C., Thomas, H. B., Lord, J., Wai, H. A., Arno, G., Beaman, G., Sergouniotis, P., Gomes-Silva, B., Campbell, C., Gossan, N., Hardcastle, C., Webb, K., O'Callaghan, C., Hirst, R. A., Ramsden, S., Jones, E., Clayton-Smith, J., Webster, A. R., Ambrose, J. C., … Ellingford, J. M. (2021). Comparison of in silico strategies to prioritize rare genomic variants impacting RNA splicing for the diagnosis of genomic disorders. *Scientific Reports*, *11*(1). https://doi.org/10.1038/s41598-021-99747-2

Saunders, C. J., Miller, N. A., Soden, S. E., Dinwiddie, D. L., Noll, A., Alnadi, N. A., Andraws, N., Patterson, M. L. A., Krivohlavek, L. A., Fellis, J., Humphray, S., Saffrey, P., Kingsbury, Z., Weir, J. C., Betley, J., Grocock, R. J., Margulies, E. H., Farrow, E. G., Artman, M., … Kingsmore, S. F. (2012). Rapid whole-genome sequencing for genetic disease diagnosis in neonatal intensive care units. *Science Translational Medicine*, *4*(154). https://doi.org/10.1126/SCITRANSLMED.3004041

Schmidt, R. J., Steeves, M., Bayrak-Toydemir, P., Benson, K. A., Coe, B. P., Conlin, L. K., Ganapathi, M., Garcia, J., Gollob, M. H., Jobanputra, V., Luo, M., Ma, D., Maston, G., McGoldrick, K., Palculict, T. B., Pesaran, T., Pollin, T. I., Qian, E., Rehm, H. L., … Lebo, M. S. (2023). Recommendations for risk allele evidence curation, classification, and reporting from the ClinGen Low Penetrance/Risk Allele Working Group. *Genetics in Medicine, 26*(3), 101036. https://doi.org/10.1016/J.GIM.2023.101036

Strande, N. T., Riggs, E. R., Buchanan, A. H., Ceyhan-Birsoy, O., DiStefano, M., Dwight, S. S., Goldstein, J., Ghosh, R., Seifert, B. A., Sneddon, T. P., Wright, M. W., Milko, L. V., Cherry, J. M., Giovanni, M. A., Murray, M. F., O'Daniel, J. M., Ramos, E. M., Santani, A. B., Scott, A. F., … Berg, J. S. (2017). Evaluating the Clinical Validity of Gene-Disease Associations: An Evidence-Based Framework Developed by the Clinical Genome Resource. *American Journal of Human Genetics*, *100*(6), 895–906. https://doi.org/10.1016/J.AJHG.2017.04.015

Tavtigian, S. V., Greenblatt, M. S., Harrison, S. M., Nussbaum, R. L., Prabhu, S. A., Boucher, K. M., & Biesecker, L. G. (2018). Modeling the ACMG/AMP variant classification guidelines as a Bayesian classification framework. *Genetics in Medicine*, *20*(9), 1054–1060. https://doi.org/10.1038/gim.2017.210

Tavtigian, S. V., Harrison, S. M., Boucher, K. M., & Biesecker, L. G. (2020). Fitting a naturally scaled point system to the ACMG/AMP variant classification guidelines. *Human Mutation*, *41*(10), 1734–1737. https://doi.org/10.1002/humu.24088

Tian, Y., Pesaran, T., Chamberlin, A., Fenwick, R. B., Li, S., Gau, C. L., Chao, E. C., Lu, H. M., Black, M. H., & Qian, D. (2019). REVEL and BayesDel outperform other in silico meta-predictors for clinical variant classification. *Scientific Reports*, *9*(1). https://doi.org/10.1038/S41598-019-49224-8

Toledo, R. A., Loureço, D. M., Camacho, C., Lindsey, S., Cerutti, J., Maciel, R. M. B., & Toledo, S. P. A. (2015). RET Y791F: alone or accompanied? *Archives of Endocrinology and Metabolism*, *59*(5), 476–477. https://doi.org/10.1590/2359-3997000000060

Walker, L. C., Hoya, M. de la, Wiggins, G. A. R., Lindy, A., Vincent, L. M., Parsons, M. T., Canson, D. M., Bis-Brewer, D., Cass, A., Tchourbanov, A., Zimmermann, H., Byrne, A. B., Pesaran, T., Karam, R., Harrison, S. M., Spurdle, A. B., Biesecker, L. G., Tayoun, A. A., Berg, J. S., … Topper, S. (2023). Using the ACMG/AMP framework to capture evidence related to predicted and observed impact on splicing: Recommendations from the ClinGen SVI Splicing Subgroup. *American Journal of Human Genetics*, *110*(7), 1046–1067. https://doi.org/10.1016/j.ajhg.2023.06.002

Whiffin, N., Minikel, E., Walsh, R., O'Donnell-Luria, A. H., Karczewski, K., Ing, A. Y., Barton, P. J. R., Funke, B., Cook, S. A., Macarthur, D., & Ware, J. S. (2017). Using high-resolution variant frequencies to empower clinical genome interpretation. *Genetics in Medicine, 19*(10), 1151–1158. https://doi.org/10.1038/GIM.2017.26

Zernant, J., Lee, W., Nagasaki, T., Collison, F. T., Fishman, G. A., Bertelsen, M., Rosenberg, T., Gouras, P., Tsang, S. H., & Allikmets, R. (2018). Extremely hypomorphic and severe deep intronic variants in the ABCA4 locus result in varying Stargardt disease phenotypes. *Cold Spring Harbor Molecular Case Studies*, *4*(4). https://doi.org/10.1101/MCS.A002733

Zhang, L., Walsh, M. F., Jairam, S., Mandelker, D., Zhong, Y., Kemel, Y., Chen, Y. B., Musheyev, D., Zehir, A., Jayakumaran, G., Brzostowski, E., Birsoy, O., Yang, C., Li, Y., Somar, J., DeLair, D., Pradhan, N., Berger, M. F., Cadoo, K., … Offit, K. (2020). Fumarate hydratase FH c.1431_1433dupAAA (p.Lys477dup) variant is not associated with cancer including renal cell carcinoma. *Human Mutation*, *41*(1), 103–109. https://doi.org/10.1002/HUMU.23900

## Appendix A: Summary of major changes from ACGS 2020 guidelines

| Section | Changes made |
|---|---|
| Introduction and throughout | Addition of CNV guidelines throughout the guidelines. |
| Introduction and reporting | Addition of section for reporting of non-coding, reduced penetrance and hypomorphic sequence variants and risk alleles. |
| Introduction | Added new gnomAD v4.0.<br>VCEP guidelines should be assessed for suitability prior to NHS use. |
| Introduction | Removed table of combining evidence for SNV classification and included information on how to use Bayesian points system. |
| Introduction | Added table with example CNVs and which guidelines to use. |
| PVS1 | Added information on truncating variants in first exon, stop loss variants, frameshifts causing a protein extension, +2T>C and +2C>T variants.<br>Added use of PVS1(RNA) for variants where RNA analysis has shown an aberrant effect on splicing.<br>Added criteria that should not be used with PVS1. |

| | |
|---|---|
| PS1 | Changed strengths in line with ClinGen VCEPs: str: pathogenic or mod: likely pathogenic.<br>Added use for splicing including variants within the same splicing motif or region as per Splicing SVI paper Walker et al 2023.<br>Added criteria that should not be used with PS1. |
| PS2 | Moved tables of examples to the appendix. |
| PS3 | Added Brnich et al 2020 guidelines for protein assays.<br>Removed use for splicing assays and moved to PVS1(RNA). |
| PS4 | Added that case and control populations should be ethnically matched where possible.<br>Added PS4 can be used with caution in late-onset/reduced penetrance disorders with low numbers in gnomAD consistent with disease prevalence where PM2 cannot be applied.<br>Added that PM3 should be used where possible for autosomal recessive disorders.<br>Added criteria that should not be “double-counted” with PS4. |
| PM1 | Added example evidence that can be used for this criterion.<br>Added criteria that should not be used with PM1. |
| PM2 | Added evidence and justification to keep PM2 strength as moderate pending new ACMG guidelines.<br>Added reference to gnomAD v4, CardioDB allele frequency app, and caution with some variants.<br>Added info on CHIP.<br>Added when not to apply PM2. |
| PM3 | Added tool to check co-occurrence.<br>Added this code should be used in preference to PS4 for autosomal recessive disorders. |
| PM4 | Added in silico tools for PM4. |
| PM5 | Variant should have a similar or greater impact on protein as predicted by REVEL, Grantham or BLOSUM62.<br>Added criteria that should not be used with PM5. |
| PP1 | Added further information on use of PP1 in recessive conditions and example. |
| PP3 | Strength should remain as supporting based on mini-impact assessment of PM2 downgrade.<br>Recommend use meta tool e.g. REVEL rather than combining multiple tools.<br>Recommend use of SpliceAI and threshold of ≥0.2. |
| PP4 | Table of phenotype specificity moved to appendix.<br>Moved use of CanVIG multifactorial data from PP5 to PP4 in line with ENIGMA guidelines. |
| PP5 | Previously allowed use for CanVIG multifactorial data moved to PP4 and PP5 deleted in line with previous ClinGen recommendation. |
| BA1 | Added new section |
| BS1 | Added CardioDB allele frequency tool. |
| BS2 | Clarified usage with examples |
| BS3 | Added new section |
| BS4 | Added new section |
| BP1 | Added use for missense variants outside of key functional domains |
| BP2 | Added new section |
| BP3 | Added new section |
| BP4 | Added SpliceAI score ≤0.1 |
| BP5 | Added caution with co-occurrence and blended phenotypes |
| BP7 | Added new section |

## Appendix B:

### Table S1: PS2/PM6 example usage

| Type of test | Parental relationships confirmed by test | Gene | Phenotype | Evidence criterion |
|---|---|---|---|---|
| Single gene followed by parental testing of variant | No | *NIPBL* | Classical clinical presentation of Cornelia de Lange including: Facial gestalt, severe global developmental delay/intellectual disability, hirsutism, upper-limb reduction defects, growth retardation and microcephaly | PM6 |
| Trio exome or genome with virtual panel analysis (e.g. DDG2P in DDD study or tiered variants in 100,000 Genomes Project) | Yes | *NIPBL* | Classical clinical presentation of Cornelia de Lange including: Facial gestalt, severe global developmental delay/intellectual disability, hirsutism, upper-limb reduction defects, growth retardation and microcephaly | PS2 |
| Gene-agnostic trio exome or genome (variants filtered by mode of inheritance) | Yes | *NIPBL* | Classical clinical presentation of Cornelia de Lange including: Facial gestalt, severe global developmental delay/intellectual disability, hirsutism, upper-limb reduction defects, growth retardation and microcephaly | PS2 |
| Trio exome or genome with virtual panel analysis (e.g. DDG2P in DDD study or tiered variants in 100,000 Genomes Project) | Yes | *NIPBL* | Severe developmental delay; no other features of Cornelia de Lange | NOT USED |
| Gene-agnostic trio exome or genome (variants filtered by mode of inheritance) | Yes | *NIPBL* | Severe developmental delay; no other features of Cornelia de Lange | NOT USED |
| Gene panel followed by parental testing of variant | No | Many examples | Early infantile epileptic encephalopathy | PM6 |
| Trio exome or genome with virtual panel analysis (e.g. DDG2P in DDD study or tiered variants in 100,000 Genomes Project) | Yes | Many examples | Early infantile epileptic encephalopathy | PS2_Moderate |
| Gene-agnostic trio exome or genome (variants filtered by mode of inheritance) | Yes | Many examples | Early infantile epileptic encephalopathy | PS2_Moderate |
| Trio exome or genome with virtual panel analysis (e.g. DDG2P in DDD | Yes | Many examples | Non-syndromic Intellectual disability | PS2_Supporting |

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| study or tiered variants in 100,000 Genomes Project) | | | | |
| Gene-agnostic trio exome or genome (variants filtered by mode of inheritance) | Yes | Many examples | Non-syndromic Intellectual disability | PS2_Supporting |

**Table S2: Examples of using phenotype specificity as evidence for PP4.**

| **Evidence Level** | **Genetic aetiology** | **Gene(s)** | **Percentage of cases explained by variants in this gene or gene panel*** | **Phenotype**<br><br>*A strong consensus supporting a clinical diagnosis of the syndrome based on the features described.* | **Functional evidence (e.g. biochemical, MRI, muscle biopsy)** |
|---|---|---|---|---|---|
| Supporting | Sotos syndrome | *NSD1* | ~90% | Facial gestalt and developmental delay/ intellectual disability or childhood overgrowth (height and/or head circumference ≥2 SD above the mean) | N/A |
| Moderate | Sotos syndrome | *NSD1* | ~90% | Facial gestalt and developmental delay/intellectual disability **and childhood overgrowth** (height and/or head circumference ≥2 SD above the mean) | N/A |
| Supporting | Kabuki syndrome | *KMT2D* and *KDM6A* | 55-80% | Facial gestalt and mild-moderate developmental delay/intellectual disability | N/A |
| Moderate | Kabuki syndrome | *KMT2D* and *KDM6A* | 55-80% | Facial gestalt, mild-moderate developmental delay/intellectual disability **and one of the following**; characteristic skeletal anomalies, fetal fingertip pads, postnatal growth deficiency, hyperinsulinism | N/A |
| Strong | Kabuki syndrome | *KMT2D* and *KDM6A* | 55-80% | Facial gestalt, mild-moderate developmental delay/intellectual disability **and one of the following**; characteristic skeletal anomalies, fetal fingertip pads, postnatal growth deficiency, hyperinsulinism | Methylation signature |
| Supporting | Gorlin syndrome | *PTCH1* and *SUFU* | 70-85% | Facial gestalt and one of the following:<br>BCC before age 30 years or multiple BCCs >5 in a lifetime, multiple jaw keratocysts, palmar or plantar pits, non-specific radiological findings | N/A |
| Moderate | Gorlin syndrome | *PTCH1* and *SUFU* | 70-85% | Facial gestalt **and/or two of the following**:<br>BCC before age 30 years or multiple BCCs >5 in a lifetime, | N/A |

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| | | | | multiple jaw keratocysts, palmar or plantar pits, non-specific radiological findings | |
| Supporting | Cornelia de Lange syndrome | *RAD21, SMC3, HDAC8* and *SMC1A* gene panel (when no *NIPBL* variant identified) | 70% | Facial gestalt and severe intellectual disability/developmental delay | N/A |
| Moderate | Cornelia de Lange syndrome | *NIPBL* or<br><br>*RAD21, SMC3, HDAC8* and *SMC1A* gene panel (if no *NIPBL* variant identified) | 70% | Facial gestalt and severe global developmental delay/intellectual disability **and one of the following**: upper-limb reduction defects, growth retardation and microcephaly | N/A |
| Strong | Hunter syndrome (MPS II) | *IDS* | | Clinical and radiological features consistent with MPS II | Deficient iduronate 2-sulfatase (I2S) enzyme activity in white cells, fibroblasts, or plasma in the presence of normal activity of at least one other sulfatase. |
| Supporting | HNF1A/4A MODY | *HNF1A/ HNF4A* | N/A | Diabetes | Improved glycaemic response when treated with sulphonylurea tablets |
| Strong | Calpainopathy | *CAPN3* | 84% for cases with severe calpain-3 protein deficiency | Clinical findings consistent with calpainopathy limb girdle muscular dystrophy and raised CK | Consistent muscle biopsy findings and immunoblot analysis identifying calpain-3 protein as absent or severely reduced |
| Moderate | CASK – related pontocerebellar hypoplasia (PCH) in an affected female | *CASK* | N/A | PCH, moderate-severe intellectual disability, progressive microcephaly | Classical *CASK* neuroimaging findings of PCH differentiating this from other cause of PCH** |
| Supporting | ATRX syndrome | *ATRX* | N/A | Severe, intellectual disability in an affected male<br>Family history compatible with X-linked recessive inheritance | HbH inclusion bodies |

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| Moderate | ATRX syndrome | *ATRX* | N/A | Facial gestalt, severe intellectual disability in an affected male, consistent genital anomalies | HbH inclusion bodies or Methylation signature |
| Strong | ATRX syndrome | *ATRX* | N/A | Facial gestalt, severe intellectual disability in an affected male, consistent genital anomalies | HbH inclusion bodies and Methylation signature |
| Supporting | Multiple Endocrine Neoplasia type 1 | *MEN1* | 80-90% for familial cases | Two endocrine tumours; parathyroid, pituitary or gasto-entero-pancreatic tract | |
| Moderate | Multiple Endocrine Neoplasia type 1 | *MEN1* | 80-90% for familial cases | Two endocrine tumours; parathyroid, pituitary or gasto-entero-pancreatic tract | Somatic loss of heterozygosity at the *MEN1* locus*** |
| Moderate | Multiple Endocrine Neoplasia type 1 | *MEN1* | 80-90% for familial cases | Two endocrine tumours; parathyroid, pituitary or gasto-entero-pancreatic tract and first degree relative also affected | |
| Moderate | Hereditary neuropathy with liability to pressure palsies | *PMP22* | 100% | Recurrent focal compression neuropathies, family history consistent with autosomal dominant inheritance and absence of diabetes | Prolongation of distal nerve conduction latencies in an individual with clinical features consistent with hereditary neuropathy with liability to pressure palsies |

*Data from GeneReviews (https://ghr.nlm.nih.gov/) accessed 01/04/2019. **Moog et al J Med Genet 2011. ***See CanVIG guidance for use of PP4 for cancer predisposition gene variants.

## Appendix C: PM2 weighting mini-impact assessment

### Introduction:

In a 2020 recommendation, ClinGen Sequence Variant Interpretation (SVI) Working Group proposed reducing the weight of PM2 to "supporting" by default (https://www.clinicalgenome.org/site/assets/files/5182/pm2_-_svi_recommendation_-_approved_sept2020.pdf). The recommendation also indicated that future adjustments to other criteria would be necessary to accommodate the change in PM2 weight. In the ACGS 2020 guidelines, there was continued support for application of PM2 at moderate level, pending a fuller revision of the ACMG guidance. A recent publication from ClinGen SVI has recommended changes to evidence weighting for missense *in silico* tools used under PP3/BP4 (Pejaver *et al.,* 2022).

### Method:

An impact assessment was undertaken by ACGS to look at the effect of downgrading PM2, along with the proposed changes to PP3 weighting on a panel of 210 likely pathogenic variants from 85 genes, all classified using PM2_mod. Included in the assessment were 105 missense, 44 splice (mostly non-canonical or in-frame variants), 34 in-frame small indels and 27 truncating

(with no NMD) variants. These variants were re-scored using PM2_sup and new PP3 weighting for missense variants based on REVEL scores from Pejaver *et al.,* (2022) and the revised classification was recorded.

**Results:**

After downgrade of PM2 to supporting and application of Pejaver (*et al.,* 2022) weighting for PP3 131/210 (62.5%) variants remained likely pathogenic, 15/210 (7%) were upgraded to pathogenic and 64/210 (30.5%) were downgraded from likely pathogenic to VUS. Of the 64 that were downgraded from likely pathogenic to VUS: 19 were in-frame variants (19/64 30%), 18 splice (18/44 41% splice), 16 truncating (16/27 59%) and 11 missense (11/105 10%).

**Conclusion:**

The new PP3 weighting for missense variants mitigated some, but not all, of the effects of the PM2 downgrade. However, for in-frame, truncating variants with no NMD and non-canonical/in-frame splice variants, further changes to the guidelines are required to uplift other codes, as there was a very significant impact on down classification for these variants Based on these results, the authors recommend that PM2 weighting is not downgraded pending further updates to the ACMG guidelines in 2024.

In addition, it is important that clear recommendations for laboratory and clinical management of variants that are downgraded due to this or other changes in guidelines are available. Loong *et al.,* (2022) includes recommended wording for laboratory reports and guidelines for clinical genetics recontact of patients for Cancer Susceptibility Genes and may also be applicable for other rare disease cases.