ACGS Best Practice Guidelines for Variant Classification 2019

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1. Introduction

In the European Union, a rare disease is defined as rare when it affects less than one in 2000 individuals. Approximately seven thousand 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 monogenic disorders caused by highly penetrant variants in a single gene. A molecular genetic diagnosis of a rare disease requires the identification of a single disease-causing variant (or bi-allelic 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 particularly increasingly in targeting treatment (Saunders *et al* 2012). However, diagnosis of a rare genetic disease can be a challenge and is contingent upon a robust understanding of the molecular aetiology of the disease. A molecular genetic diagnosis underpins robust disease classification, provision of prognostic information, accurate risk prediction for relatives, and importantly can indicate the most appropriate treatment(s), inform access to clinical screening, prevention strategies or clinical trials and facilitate access to support services and patient-led support groups.

Historically, genetic testing focused on the analysis of one or a small number of genes indicated by the patient's phenotype, but the advent of next generation sequencing technology has revolutionised the scale at which genetic testing can be performed enabling the analysis of many more genes within the same assay. Large gene panel tests (>100 genes) and whole exome sequencing are routinely available in UK clinical diagnostic laboratories and whole genome sequencing, first available through the 100,000 Genomes

Project in England, will be commissioned for mainstream clinical care within the NHS in England in the near future. Deciphering which, if any, of the observed variants are disease-causing is challenging as each human genome has 3-4 million variants (compared to the reference human genome sequence). Only a minority are causative of monogenic disease; most are part of normal human variation or may contribute to an increased or decreased risk of multi-factorial disease. The gnomAD database (http://gnomad.broadinstitute.org/) currently includes 17.2 million variants identified by exome sequencing of 125, 748 individuals and 261.9 million variants identified through genome sequencing of 15,708 individuals who were part of various disease-specific and population genetic studies, (Karczewski *et al* BioRxiv 2019 https://doi.org/10.1101/531210), but we do not yet have a comprehensive catalogue of global genetic variation. The focus of these guidelines is the classification of highly penetrant protein-coding variants. Inferring pathogenicity of non-coding variants is more complex, but will need to be addressed as a standard of practice in the future.

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* Genetics in Medicine 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 mis-diagnosis can be harmful not just for the proband but also their relatives whose clinical management is altered as a consequence of cascade testing.

Further development of the ACMG/AMP guidelines is being undertaken through the US ClinGen Sequence Variant Interpretation (SVI) Working Group (https://www.clinicalgenome.org/working-groups/sequence-variant-interpretation/). Their goal is to support the refinement and evolution of the guidelines. It was recognised by Richards *et al* (2015) that more focused guidance regarding the classification of variants in specific genes is required given that the applicability and weight assigned to certain criteria may vary by disease and gene. A number of disease-specific variant expert panels have been established and are generating disease/gene specific guidelines (see Kelly *et al* 2018 for *MYH7*-specific guidelines). Work is also underway to consider interpretation and reporting of variants with reduced penetrance.

High quality, accurate variant interpretation requires scientific knowledge of the gene structure, function, previously identified variants and disease mechanism in addition to comprehensive clinical knowledge of the patient and their families' medical history. The UK's National Health Service (NHS) provides a unique opportunity to integrate curated genotype and phenotype information within a nationally developed database. On 4th November 2016 a group of NHS clinical scientists and clinical geneticists met to discuss the implementation of the ACMG guidelines within the UK (see workshop report from the PHG Foundation).

A consensus statement was issued on 11th November 2016 by the Association for Clinical Genomic Science (see ACGS consensus statement or Appendix 1). It recommended

adoption of the ACMG guidelines for germline variant classification and interpretation in UK diagnostic genetic laboratories performing testing for rare disease and familial cancers.

A "Train the Trainers" workshop was held in February 2017 and attended by representatives from 24 regional genetics centres across England, Scotland, Wales and Ireland. The aim of the workshop was to plan the implementation of the ACMG guidelines in a co-ordinated way in order to achieve accurate usage and hence consistent use of the guidelines across and within laboratories. Monthly WebEx meetings for rare disease and familial cancer predisposition were established in 2017 to facilitate variant interpretation for SNVs and indels through multi-disciplinary case-based discussion and provide an opportunity for reviewing updates to the guidelines.

Please note that these guidelines are intended for general use in classifying variants in patients with rare diseases. Disease-specific guidelines are being developed for disorders where different evidence thresholds are required, for example familial cancer predisposition and inherited cardiac conditions.

2. 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, mutational mechanism (e.g. haploinsufficiency, dominant negative), protein structure/function and the strength of the gene-disease relationship (Strande *et al* Am J Hum Genet 2017). With the exception of the patient's phenotype data, most of this information can be obtained from the published literature/databases by a clinical scientist who can also collate the required population data and *in silico* predictions of variant effect.

The level of detailed phenotype data provided with the laboratory referral depends upon the testing scenario. If the test is for a single gene or small panel of genes where the patient's phenotype is highly specific for that disorder, then detailed phenotype data is not required for variant interpretation. In other settings, for example specialist services for heterogeneous disorders, laboratories require the submission of a defined phenotypic data set for use by clinical scientists to decide appropriate testing strategies and/or for variant interpretation. 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 group meeting, video or teleconference, e-mail correspondence or a telephone conversation between a member of the referring clinical team and a laboratory scientist responsible for the case.

The key question for the referring clinical team in an MDT discussion is "Does this patient's phenotype fit this gene-disease association?" If so, what is the strength of the evidence to support the variant classification? Tools to evaluate this aspect of the variant classification

process are in development, for example the Summative Assessment tool within DECIPHER (https://decipher.sanger.ac.uk/). 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 ACMG/AMP guidelines that incorporate information regarding the patient's phenotype; the *de novo* variant assessment, PS2/PM6, and the phenotype specificity, PP4.

The *de novo* variant evidence assessment is recorded using the PS2 and PM6 criteria. PS2 is used when both parental relationships have been confirmed, either through trio exome/genome analysis or using a panel of informative genetic markers, and PM6 is used if testing for one or both parental relationships has not been undertaken. PS2 and PM6 can only be used if the patient's phenotype is consistent with the disease gene association. The level of evidence applied is determined by the phenotypic specificity. The nature of the testing strategy should also be considered when applying PS2 and PM6 (see Table 1 for examples of how to apply these evidence criteria with consideration given to the testing strategy employed). 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 NEJM).

Table 1: Examples of the use of *de novo* evidence according to the type of test undertaken and the specificity of the phenotype. Note that trio exome or genome sequencing would reveal non-biological parental relationships. This table should be used in conjunction with the points-based system developed by the ClinGen Sequence Interpretation Group which indicates modification of the evidence strength given multiple reports of *de novo* events (see https://www.clinicalgenome.org/site/assets/files/8490/recommendation_ps2_and_pm6_acmgamp_critiera_version_1_0.pdf).

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

PP4 can be used as a supporting piece of evidence when the patient's phenotype in its entirety is consistent with a highly specific genetic aetiology. In some situations it is considered appropriate to use this evidence criterion at a moderate or strong level after MDT discussion (see Table 2 below for examples). In order to use PP4 it is essential that (a) all the known genes associated with the disorder have been analysed using a highly sensitive method (or methods) appropriate for the reported types of likely pathogenic/pathogenic variants and (b) variants in these known genes explain the majority of cases with that clinical diagnosis.

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 drug enzyme 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.

Although the ACMG/AMP guidelines include the inclusion of functional evidence from enzymatic assays performed on patient tissue within the PS3 criterion, such data provides support at the gene rather than variant level, and may be considered more appropriate as evidence supporting the phenotype specificity. For these reasons we recommend that only functional evidence at the level of the variant is utilised within the PS3 criterion.

Table 2: Examples of using phenotype specificity as evidence for PP4.
*Data from GeneReviews (https://ghr.nlm.nih.gov/) accessed 01/04/2019. **Moog *et al* J Med Genet 2011

Evidence Level	**Genetic aetiology**	**Gene(s)**	**Percentage of cases explained by variants in this gene or gene panel***	**Phenotype** *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
Supporting	Gorlin syndrome	*PTCH1* and *SUFU*	70-85%	Facial gestalt and one of the following: 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**: BCC before age 30 years or multiple BCCs >5 in a lifetime,	N/A

				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 *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**
Moderate	ATRX	*ATRX*	N/A	Facial gestalt, severe	HbH inclusion

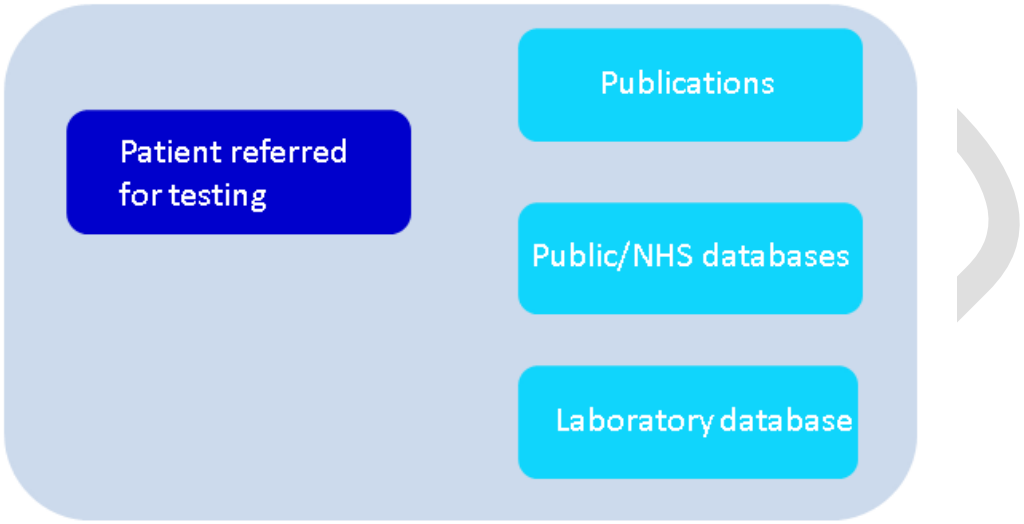
	syndrome			intellectual disability in an affected male, consistent genital anomalies	bodies
Supporting	ATRX syndrome	*ATRX*	N/A	Severe, intellectual disability in an affected male Family history compatible with X-linked recessive inheritance	HbH inclusion bodies
Supporting	Multiple Endocrine Neoplasia 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

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 frequently, phenocopies are common and other genetic and environmental factors influence penetrance and phenotype in gene carriers and non-carriers. As noted above, different evidence thresholds may be required in these disorders and disease-specific guidelines are being developed for familial cancers and inherited cardiac conditions. We note that 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.

3. **Variant classification: Supplementary notes for use of the ACMG evidence criteria**

The assessment of a variant should include phenotype data from all patients currently identified with the variant; the patient referred for testing, previous patients tested in the laboratory, published literature and information from variant databases (see Figure 1).

Figure 1: The evidence for a variant classification is assessed across all patients for which information is available.



The framework developed by the ACMG team utilises a series of evidence criteria in support of a pathogenic (P) or benign (B) classification. These are described in tables 3 and 4 of the publication by Richards *et al* (2015). The different types of evidence (functional, genetic, population, *in silico* etc.) are stratified according to the level of evidence (supporting, moderate, strong, very strong) and a pathogenicity classification (pathogenic, likely pathogenic, VUS, likely benign or benign) assigned according to a set of "combining criteria" according to Table 5 in Richards *et al* (2015).

The ACMG guidelines have been transformed into a quantitative Bayesian framework by Tavtigian *et al* (2018). Testing of this framework against the "combining criteria" identified two inconsistencies. First, likely pathogenic rule (i) (one very strong plus one moderate evidence of pathogenicity) gave a posterior probability of 0.994 which is equivalent to pathogenic rules (iiia, iiib and iiic). Second, pathogenic rule (ii) (at least two strong criteria in favour of pathogenicity) gave a posterior probability of 0.975 which is weaker than the other pathogenic rules which yield a posterior probability of > 0.99. The ACGS recommends that those variants for which there is one very strong plus one moderate criteria in favour of pathogenicity are classified as pathogenic. Most frequently these are loss of function variants predicted to result in nonsense mediated decay that have not been reported in the gnomAD database. Prior to implementation of the ACMG guidelines they would have been reported as pathogenic. Note the essential requirement that there is robust evidence to support loss of function as a known mechanism for the disease. Likewise we recommend that those variants with evidence for only two strong criteria (posterior probability of 0.975)

are classified as likely pathogenic (requiring an additional one moderate or two supporting criteria to classify as pathogenic with a posterior probability of 0.994).

Table 3 (below) 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 Jarvik & Browning (2016). 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.

A set of supplementary slides has been developed (Appendix 2) to support use of these guidelines.

Table 3: Supplementary information for classifying pathogenic (P) or benign (B) variants

Evidence criteria (level) supplementary notes
PVS1 – (Very Strong) null variant (nonsense, frameshift, canonical ±1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where LOF is a known mechanism of disease. 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 (Tayoun *et al* 2018). 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 and PVS1_Very strong 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 2). Note that caution is required when interpreting 3' nonsense or frameshift variants predicted to escape nonsense mediated decay and consensus spice donor/acceptor site variants predicted to lead to in frame deletions. 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 risk of familial breast cancer (Mazoyer *et al* 1996). Nor does the *BRCA1* c.594-2A>C slice acceptor site variant (de la Hoya *et al* 2016).
PS1 – (Strong) Same amino acid change as a previously established pathogenic variant regardless of nucleotide change. This criterion can be used if there is sufficient evidence for pathogenicity for the same missense variant (ie an amino acid change) caused by a **different** base substitution. For example the previously reported variants 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 may also be used in two other scenarios. First, at a moderate level for initiation codon variants where a different nucleotide substitution affecting the initiation codon has been classified as (likely) pathogenic. Second, at a supporting level for splicing variants where a different nucleotide substitution has been classified as (likely) pathogenic and the variant being assessed is predicted by *in silico* tools to have a similar or greater deleterious impact on the mRNA/protein function.
PS2 – (Strong) 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 typically contains between 1-2 *de novo* non-synonymous variant 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. Please see Table 1 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 (see

https://www.clinicalgenome.org/site/assets/files/8490/recommendation_ps2_and_pm6_acmgamp_critiera_version_1_0.pdf). Please note that the same, not a higher, level of phenotypic specificity should be applied when using this points-based system for variants reported in multiple cases.

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

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, or investigation of putative splicing variants through mRNA analysis from patient material or use of a minigene splicing assay. Where functional data, for example from biochemical testing, provides support at the gene rather than variant level this should be incorporated within the phenotypic specificity criterion (PP4). *In silico* studies, including protein modelling, are not considered sufficient evidence for this criterion (but may be incorporated in PM1 evidence). Note that evidence from functional studies must be carefully assessed to determine the data quality, reliability and hence degree of confidence in the results. For example a test that is carried out in a certified diagnostic laboratory, has been replicated in a second centre, or a variant that has undergone multiple functional assessments using different methodologies would provide greater confidence that the variant has a damaging effect upon the gene product. *In vitro* transfection studies which result in over expression of the protein product and cell studies investigating subcellular location and or function where the physiological relevance of the particular finding(s) has not yet been firmly established should be treated with caution.

PS4 – (Strong) 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 this may not be appropriate when there are many cases of the disorder included in the data set, for example in cardiovascular diseases.

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,* **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. Absence from the gnomAD database also allows use of PM2 at moderate level, i.e. both PS4 (moderate or supporting) and PM2 can be used.

PM1 – (Moderate) 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 3) or can be generated using this link). *In silico* protein modelling data can be included as supporting evidence.

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 are most common cause of collagen triple helix phenotypes as the glycine in the Gly-X-Y repeat is critical for correct structure, and cysteine or histidine substitutions in C2H4 zinc fingers such as *GLI3*.

PM2 – (Moderate) Absent from controls (or at extremely low frequency if recessive) in Exome Aggregation Consortium.

It is important to check that the variant position is covered to sufficient read depth in ExAC (or gnomAD). To check in ExAC see this link. The gnomAD coverage data is available from https://console.cloud.google.com/storage/browser/gnomad-public/release/2.1/coverage. Be aware that indels are less readily identified by next generation sequencing and ascertain whether other indels have been detected within the region.

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

This applies to previous cases with either a pathogenic or likely pathogenic variant confirmed *in trans* with the variant being assessed. Note that if there are multiple observations of the variant *in trans* with other pathogenic variants then this evidence can be upgraded to strong (Richards *et al* 2015). A points-based system is under development by the ClinGen Sequence Variant Interpretation group (using the same scale as for PS2). Homozygous occurrences can be included but are reduced by one evidence level to take into consideration the greater prior probability of non-independent allelic segregation.

PM4 – (Moderate) 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 and would also apply to a deletion of a small in-frame 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.
PVS1 is used for out of frame exon deletions and larger in-frame exon deletions that remove a significant proportion of a gene. Please note that PM4 should not be applied if PVS1 is used (Abou Tayoun *et al* 2018). There is no fixed definition of small/large as the impact of a deletion will depend on the size of a gene and the gene architecture (including the impact of a deletion on functional domains or regulatory elements). Greater care should be taken with apparent in-frame exonic insertions/duplications since it is harder to predict their impact at the protein level, and their precise location and orientation may not be known unless demonstrated by whole genome sequencing.

PM5 – (Moderate) Novel missense change at amino acid residue where a different missense change determined to be pathogenic has been seen before
Interpret as "missense change at amino acid residue where a different missense change determined to be pathogenic has been seen before" ie the variant does not need to be novel. The previously identified missense variant can be classified as pathogenic or likely pathogenic but if the variant is classified as likely pathogenic and there is only one case reported then we recommend use at supporting level.

PP1 – (Supporting) Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease.
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.

PP2 – (Supporting) 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 constraint for the region encompassing the variant, not just across the entire gene. The DECIPHER database shows regional constraint within the protein view missense constraint track (see Figure 3). New models for calculating regional constraint are being developed (Traynelis et al 2017; Havrilla et al 2019; Samocha et al 2019).

PP3 – (Supporting) Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.).
In silico splicing prediction tools can be used as evidence to suggest a significant impact on splicing potential for splice site variants outside the canonical splice acceptor (-1 and -2) and donor (+1 and +2) regions.

Variants affecting the last base of an exon or +5 have an increased prior probability of aberrant splicing. PP3 may be used at a supporting level for variants where MaxEntScan predicts >15% reduction compared to reference allele AND SpliceSiteFinder-Like predicts >5% reduction. Note that Max-Ent only predicts aberrations in the Cartegni region (ie 3 bases into exon, ~14 bases into intron) and does not predict native GC splice donor sites (use SpliceSiteFinder-Like for these).

PP3 may also be applied where splice prediction algorithms indicate the introduction of a cryptic splice site with the potential to cause aberrant splicing, eg. the introduction of a 3' (acceptor) site in an intron.

PS3 can 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 prediction is not independent evidence.

For predicting the impact of missense variants it is likely that a meta-predictor tool (e.g. REVEL, Ioannidis *et al* 2016 or GAVIN, van der Velde *et al* 2017) will replace the use of multiple prediction tools that each assess overlapping subsets of the evidence. These tools may be used to generate evidence for PP3 or BP4 (or not used if within a "grey area" where neither apply). Threshold scores for use with meta-predictor tools have not yet been defined but for REVEL they are likely to be around ≥0.7 for PP3 and ≤0.4 for BP4. It is important that any in-house validation studies use a suitably powered set of variants not included in the training sets used to develop the tool.

PP4 – (Supporting) 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.

In certain circumstances where the presenting phenotype is highly specific/pathognomonic of a single genetic aetiology, it may considered appropriate to use this evidence criterion at a moderate or strong level after MDT discussion (see Table 2 for examples). The key consideration with this evidence criteria is the specificity of the phenotype and caution should be exercised when considering phenotypic features which are specific to a disorder that is genetically heterogeneous. 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.

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.

PP5 – (Supporting) Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation.
The ClinGen Sequence Variant Interpretation group recommends that this criterion is not used (Biesecker and Harrison, 2018). This also applies to BP6.

Exceptional cases: For genes conferring susceptibility to common cancers, sufficient burden of evidence for classification can typically only be derived from analyses involving large series of enriched cases. The vast majority of such datasets currently reside in large commercial testing laboratories and have not yet been made widely available. Therefore, as an interim measure, in anticipation of collaboration of commercial laboratories within expert groups, we would sanction use of PP5 where a recent classification has been made by such a laboratory of a variant in such a cancer susceptibility gene.

BS1 – (Strong) 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.*

BP1 – (Supporting) Missense variant in a gene for which primarily truncating variants are known to cause disease.
This criterion 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).

Figure 2: Use of PVS1 and PM4 for stop loss variants (courtesy of Kevin Colclough, Royal Devon & Exeter NHS Foundation Trust and including part of the PVS1 decision tree re-drawn from https://www.biorxiv.org/content/early/2018/05/09/313718) NMD=nonsense mediated decay; NSD=nonstop mediated decay

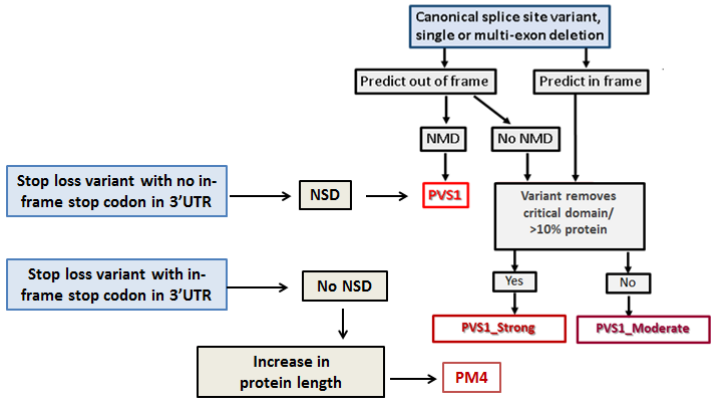
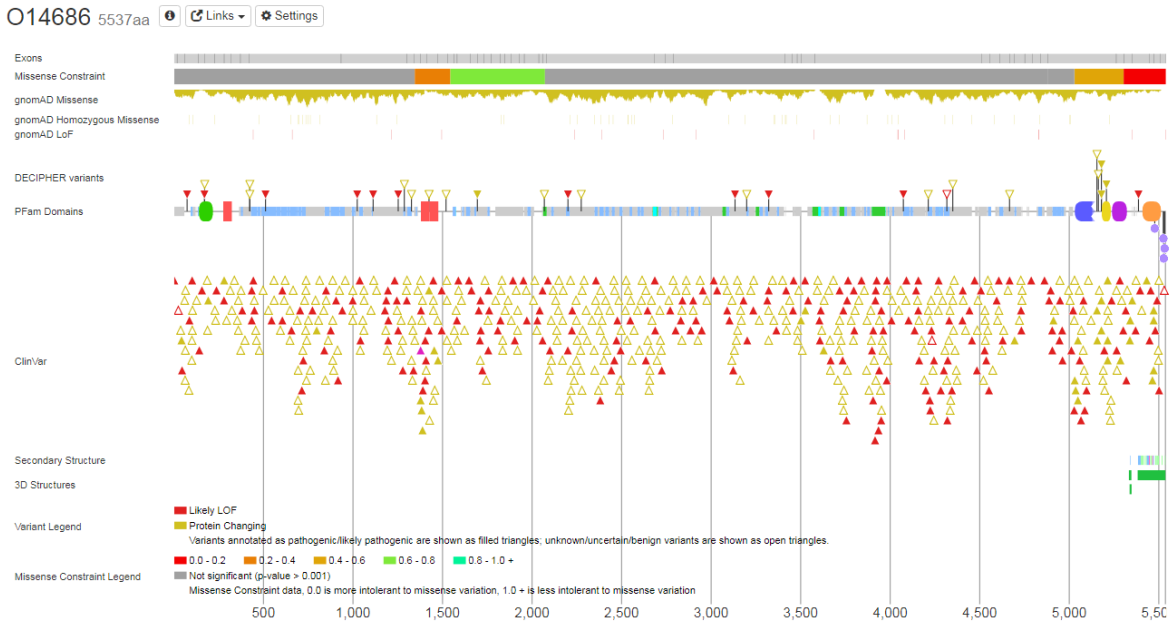


Figure 3: Example plot of *KMT2D* functional domains, ClinVar variants, proxy population/benign variants and missense constraint from DECIPHER (https://decipher.sanger.ac.uk/gene/KMT2D#overview/protein-info).



4. **Reporting the variant classification**

The aim of genomic testing for a patient with a rare disease of unknown cause is to provide a genetic diagnosis by identifying a (likely) disease-causing variant (or bi-allelic variants in autosomal recessive conditions). The genetic analysis may involve the pathogenic assessment of one or multiple variants but the clinical diagnostic report will only describe those that are relevant, or have likely or possible relevance to the patient's clinical presentation. Results included within a clinical diagnostic report will form part of the patient's clinical record and should be unambiguous to a non-specialist.

Variants are classified as "pathogenic", "likely pathogenic", "uncertain significance", "likely benign" or "benign" with respect to a disease and inheritance pattern. The variant classification must be included within the main body of the clinical diagnostic report together with the gene-disease association and the mode of inheritance. The evidence supporting the variant classification can be included in an appendix to the report (see example in Figure 4).

Next generation sequencing of large gene panels, a whole exome or whole genome will identify multiple variants of uncertain significance. For large gene panels the more genes tested, the higher the likelihood of finding spurious variants not causal of the patient's phenotype. It is important to remember that not all types of variants can be detected by next generation sequencing and there are still many gene-disease associations to be discovered. The consequences of a mis-diagnosis have wider family implications beyond the proband.

The Association for Clinical Genomic Science (ACGS) Practice guidelines for Targeted Next Generation Sequencing Analysis and Interpretation recommend that variants of uncertain significance are only reported within the main body of the report if there is further genetic testing or clinical investigation(s) (which may include a trial of treatment that is specific for the genetic aetiology e.g. biotin in a patient with biallelic *BTH* variants), that is likely to re-classify the variant as either "likely pathogenic" or "likely benign". It is important to consider the context in which genomic testing is undertaken. For example rapid testing of cancer predisposition genes such as *BRCA1* or *BRCA2* to determine pharmaceutical treatment options for the affected patient might report only "pathogenic" or "likely pathogenic" variants, in contrast to testing that seeks to inform prophylactic surgery options for the proband and their relatives where further testing/investigation of a variant of uncertain significance may be appropriate. However, there may also be situations in which an MDT discussion concludes that there is clinical utility in reporting a variant of uncertain significance where it may be impossible to obtain sufficient evidence to reach a variant classification of "likely pathogenic" due to lack of family member samples, but all the available clinical, gene-level and variant-level evidence supports the likely diagnosis. For example, a child with a developmental disorder where no cascade or prenatal testing is required, but a likely diagnosis will provide access to support services for the patient and their family.

The ACGS recommends that unless there are specific reasons to the contrary (examples as described above), a statement is included in a report for a variant of uncertain significance. For example, **"This result does not confirm a genetic diagnosis of *disorder X* and should not be used in isolation for clinical decision making"** or **"The clinical significance of this variant is uncertain (see Appendix 1) and it should not be used in isolation for clinical decision making".**

Figure 4. Evidence for *GLUD1* p.(Arg318Ser) classification

Variant details

Gene	Zygosity	Inheritance	HGVS description	Location: GRCh37 (hg19)	*Classification
GLUD1	Heterozygous	Not known	NM_005271.3:c.954A>C p.(Arg318Ser)	Chr10:g.88820777T>G	Likely pathogenic

Evidence for variant classification using ACMG/AMP guidelines (Evidence code_ level) (Richards et al 2015 Genet Med)
▪ The p.Arg318 residue is located in the catalytic domain of the Glutamate Dehydrogenase (GDH) protein (PM1_Moderate). ▪ The variant has not been reported in the gnomAD database (123,130 individuals) (PM2_Moderate). ▪ Two different missense variants, p.(Arg318Lys) and p.(Arg318Thr), have been reported in patients with hyperinsulinism-hyperammonemia syndrome (Miki *et al* 2000 PMID 10636977 and Hallsdorsdottir et al 2000 J Endocr Genet). A *de novo* p.(Arg318Lys) variant was also found in a patient tested in this laboratory (PM5_Moderate). ▪ The *GLUD1* gene has a low rate of benign missense variation as evidenced by a significant (z= 4.89) ExAC constraint score (PP2_Supporting). ▪ The p.Arg318 residue is conserved across 21 species to zebrafish. The p.(Arg318Ser) variant is predicted by SIFT, PolyPhen and AlignGVGD to have a deleterious effect upon protein function (PP3_Supporting). ▪ *GLUD1* pathogenic variants are the only known cause of hyperinsulinism-hyperammonemia syndrome (PP4_ Supporting).

5. Reclassification of variants

Variant data and relevant associated information must be stored within the laboratory in a way that allows reclassification if required. Sharing of variant data on a global scale in a manner that conforms to UK information governance requirements is a goal supported by the ACGS and BSGM (British Society for Genetic Medicine). The DECIPHER database (https://decipher.sanger.ac.uk/) hosts an NHS consortium project to allow sharing of variant data in a restricted manner. This allows member laboratories to identify conflicting classifications for the same variant to enable submitters to discuss the most appropriate classification based on the available evidence.

Reassessment of a variant that results in reclassification may be prompted by the publication of new knowledge regarding the variant (or gene-disease 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.

We propose that 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 this is feasible) that generated the original classification status to ensure consistency across centres. The new classification data and the basis for this classification should be placed in a publicly accessible database 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 mastectomy, this decision should be documented (through an MDT) and communicated to the patient's clinical team(s) as quickly as possible. If such a variant is identified, in addition to the above two actions, this variant classification information should be disseminated rapidly (within one week) to all other diagnostic genetics laboratories within the UK using a designated secure NHS e-mail address for each laboratory. With the implementation of the NHS England Genomic Medicine Service a more regulated method for data-sharing will be created in the near future, but ultimately it is the professional responsibility of the Clinical Genomic community to ensure data is shared responsibly for improved patient care.

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Appendix 1: ACGS Consensus Statement 11/11/2016

Consensus statement on adoption of American College of Medical Genetics and Genomics (ACMG) guidelines for sequence variant classification and interpretation 11/11/2016

Headline Consensus Statement

ACGS recommends adoption of the ACMG guidelines (Richards, 2015) for sequence variant classification and interpretation in UK diagnostic genetic laboratories carrying out testing for rare disease and familial cancers.

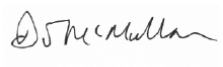
Background

Classification and interpretation of genomic variation is a highly complex discipline and in the clinical setting the need for accuracy and consistency is essential to maximise patient benefit and minimise harm. The revolution in genomic technology has led to increased routine detection of novel variants in a rapidly increasing number of novel disease genes. ACMG recently attempted to address the challenges faced by devising a detailed systematic framework for sequence variant interpretation which has now been widely adopted in the US and many European centres. Furthermore, expert panels are being formed as part of the ClinGen resource consortium to develop gene and disease specific criteria to supplement the original framework.

A Workshop hosted by ACGS was held at Austin Court, Birmingham on 4th November 2016 to reach an expert consensus view on adoption of the ACMG guidelines by the UK clinical genomics community. 70 delegates attended representing most Regional Genetics services (lab and clinical teams) with additional invited representation from all BSGM constituent groups, NHS-E (Genomics Implementation Unit), Genomics England, UKNEQAS, UKGTN, DECIPHER/DDD, HEE (Genomics Education Programme) and PHG-Foundation. The agenda included presentations on NEQAS assessment of consistency in interpretation, experiences in early adoption of ACMG guidelines, harmonisation with CNV classification, frameworks for clinical/phenotypic classification, and integration of ACMG framework into DECIPHER, together with breakout group discussions. A summary report of the Workshop is being prepared by PHG-Foundation for circulation in early 2017.

There was clear consensus agreement that the UK clinical genomics community should adopt ACMG sequence interpretation guidelines as soon as possible. ACGS, with support from HEE, will develop a multi-disciplinary training approach starting with a train-the-trainer event in early 2017. This will catalyse centre and region based adoption and also identify UK specific issues which may augment the guidelines when they are built into UK Best Practice guidelines. ACGS and BSGM annual meetings in 2017 will incorporate updates on progress with these important initiatives. In addition ACGS will offer to support further guideline development with ClinGen and ACMG via distributed variant data analyses.

Signed by: **Dom McMullan | Chair, ACGS**



Endorsed by: **Professor Bill Newman | Chair, BSGM**

Appendix 2: Supplementary slides

PVS1	_VSTR _STR _MOD

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

- If there is no evidence that LOF variants cause disease, PVS1 should **NOT** be applied at any strength level (may mean using no variant type code)
- Use caution if at 3' end or down-steam of the most 3' truncating variant
    - If the stop codon occurs in the last exon or in the last 50bps of the penultimate exon, NMD might not be predicted
- Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact
    - **Important to identify predicted effect on protein**
        - https://web.expasy.org/translate/
- Use caution in the presence of multiple transcripts
- Do not use in conjunction with PM4 or PP3
- Use PP3 for variants outside the canonical splice acceptor (-1 and -2) and donor (+1 and +2) regions that are predicated to impact on splicing
- Can be used at **very strong**, **strong** or **moderate** depending on the variant type, location in the gene or evidence for the likelihood of a true null effect.
- See LOF decision tree and criteria (Tayoun *et al* 2018)

PS1	_STR _MOD _SUP

Same amino acid change as a previously established pathogenic variant regardless of nucleotide change

- Key word here is **same** amino acid
- This does not apply to different amino acid changes – for this use PM5
- Applied if the **nucleotide change is different** (if nucleotide change is the same see PS4)
- Example: your patient's variant is p.Val12Leu (c.34G>**T**) and there is a previously reported variant p.Val12Leu (c.34G>**C**)
    - The other variant must be classified as likely pathogenic or pathogenic
- Generally for missense variants but may also be used at **moderate** for initiation codon variants.
- Beware of changes that impact splicing rather than at the amino acid/ protein level
- May also be used at **supporting** if *different* intronic change with the same or more severe splicing effect w.r.t the predicted impact on the mRNA/protein
  e.g. c.1185+5G>**T**, compared to c.1185+5G>**C**

PS2

_VSTR
_STR
_MOD
_SUP

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

- If variant identified by trio exome or genome sequencing then maternity and paternity already should have both been confirmed
    - A cautious approach is still recommended since every exome typically contains 1-2 *de novo* non-synonymous variants
- Confirmation of paternity only is insufficient
    - Egg donation, surrogate motherhood, errors in embryo transfer etc. can contribute to non-maternity
- If identity is assumed but not confirmed use PM6
- Genotype must be consistent with the phenotype
    - 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 1 for examples)
    - Can be difficult to assess in the laboratory setting without complete clinical information and therefore MDT/clinical input may be necessary
    - Do not use if inconsistent with phenotype
- X-linked conditions

  If an X-linked variant occurs *de novo* in an unaffected carrier mother, and family history is consistent – i.e. she has no affected brothers/other male relatives apart from her affected son(s) – *de novo* criteria may be applied despite the fact that she is unaffected
- Autosomal recessive conditions

  For a *de novo* occurrence in a gene associated with an autosomal recessive condition without an additional pathogenic/likely pathogenic variant identified, the strength of evidence should be decreased by one level
- Mosaicism

  For cases with apparent germline mosaicism (multiple affected siblings with both parents negative for the variant), paternity/maternity must be confirmed in order for *de novo* criteria to apply
- New points based system to determine strength of evidence: https://clinicalgenome.org/site/assets/files/3461/recommendation_ps2_and_pm6_acmgamp_critiera_version_1_0.pdf

Table 1. Points awarded per de novo occurrence

Phenotypic consistency	Points per Proband	
	Confirmed de novo	Assumed de novo
Phenotype highly specific for gene	2	1
Phenotype consistent with gene but not highly specific	1	0.5
Phenotype consistent with gene but not highly specific and high genetic heterogeneity*	0.5	0.25
Phenotype not consistent with gene	0	0

*Maximum allowable value of 1 may contribute to overall score

Table 2. Recommendation for determining the appropriate ACMG/AMP evidence strength level for de novo occurrence(s)

Supporting (PS2_Supporting or PM6_Supporting)	Moderate (PS2_Moderate or PM6)	Strong (PS2 or PM6_Strong)	Very Strong (PS2_VeryStrong or PM6_VeryStrong)
0.5	1	2	4

PS3	_STR _MOD _SUP

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

- Functional studies for a **gene variant** that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well established and can include:
  - *In vitro* functional assays (e.g. reporter genes for transcription factors or saturation genome editing to assay missense variants at scale)
  - mRNA analysis from patient samples for suspected splicing variants - can use at **strong**
  - Minigene analysis of a splicing variant
- Evidence must be carefully assessed to determine the data quality, reliability and hence degree of confidence in the results
- Can be used at **strong** , **moderate** or **supporting** depending on strength of evidence (e.g. if only one reference available use at lower strength)
- *In silico* studies including protein modelling **cannot** be used – but maybe incorporated into evidence for PM1
- Where functional data provides support at the gene rather than variant level (e.g. biochemical analysis) this should be incorporated within the phenotypic specificity criterion PP4

PS4	_STR _MOD _SUP

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

- Relative risk or odds ratio, as obtained from case-control studies, is >5.0, and the confidence interval around the estimate does not include 1.0
- Case-control study data is rarely available for rare diseases, therefore:
  - Use at **moderate** level if the same variant has been previously identified in multiple (two or more) unrelated affected individuals and has not been reported in gnomAD (or at extremely low frequency if AR)
  - Use at **supporting** level if the same variant has been previously identified in one unrelated affected individual and has not been reported in gnomAD (or at extremely low frequency if AR)
  - Use at **supporting** level if the same variant has been previously identified in multiple unrelated individuals but is NOT absent in controls
  - **The patient phenotype must be consistent with the known disease spectrum**
- In practice this is most applicable to autosomal dominant disorders (for autosomal recessive disorders use PM3)
- Both PS4 and PM2 can be used together

PM1

_STR
_MOD
_SUP

Located in a mutational hot spot and/or critical and well-established functional domain (e.g. active site of an enzyme) without benign variation

- Only applies to **missense variants**
- Several tools are available:
  - https://decipher.sanger.ac.uk/genes plots functional domains, gnomAD variants, ClinVar & DECIPHER variants and missense constraint
  - https://github.com/rdemolgen/snippets/tree/master/PM1_plots from Exeter plots HGMD variants, Consurf scores and gnomAD variation.
  - http://subrvis.org plots functional domains or exons and calculates a subRVIS score which indicates how tolerant or intolerant a region is to functional variation
- Use above plots or gnomAD to assess benign missense variation in region
  - Consider using at **supporting** if in functional domain but some benign missense variation is present – use judgement about the degree of variation that might be tolerated. If recessive gene with low missense variation can keep at **moderate**
- A mutational hotspot can be considered if pathogenic variants are observed at a high frequency in one or several nearby residues
- Use at **strong** for very specific residues that are critical for protein structure or function (see Table 3 for examples)
- *In silico* protein modelling data can be included as **supporting** evidence

PM2

_MOD

Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium

- gnomAD contains exome and genome data from unrelated individuals
  - Individuals known to be affected by severe paediatric disease, as well as their first-degree relatives, are not included
  - It includes data from 7 populations (each represented to differing degrees) and a smaller 'other' group where a population is not assigned
  - If known, it is appropriate to consider ethnicity of a patient in light of population-specific polymorphisms and the fact that your patient's ethnic group may not be well represented
- The variant must be covered to a sufficient read depth/quality in database
  - There is an interactive IGV.js visualisations to show the reads used to call the variant
  - E.g. if AD variant seen in 1/240,000 but is poor quality/filtered call, use PM2
- Insertions/deletions may be poorly called by NGS, therefore be cautious of using this code if absent in gnomAD
- There is no cut-off for recessive genes, but as a guide consider using PM2 if the frequency of the variant is below the expected carrier frequency

PM3	_STR _MOD
For recessive disorders, detected *in trans* with a pathogenic variant	
• Requires testing of parents (or offspring) to confirm phase • Can be used if detected *in trans* with (likely)pathogenic variant in another patient with the disease either in the literature or in the laboratory • Can use for homozygous variants but downgrade by one evidence level (ClinGen SVI points-based system is under development) • Can be upgraded to a **strong** level of evidence if there are multiple observations of the variant *in trans* with (likely) pathogenic variants • If the second variant is instead *in cis*, consider using **BP2** • In the context of dominant disorders, the detection of a variant *in trans* with a pathogenic variant can also be considered supporting evidence for a benign impact (**BP2**)	

PM4	_MOD _SUP
Protein length changes as a result of in-frame deletions/insertions in a non- repeat region or stop-loss variants	
• Used for in-frame deletions or insertions and also applies to a deletion of a small in-frame exon (i.e. not for frameshifts predicted to escape NMD) • If present in a repeat region consider using **BP3** • **PVS1** is used for out-of-frame exon deletions and larger in-frame exon deletions that remove a significant proportion of a gene ○ No fixed definition of small/large as the impact of a deletion will depend on the size of a gene, the gene architecture and the impact on functional domains or regulatory elements • Care should be taken with apparent in-frame exonic ins/dups since it is harder to predict their impact at the protein level, and their precise location and orientation may not be known • Stop loss variants are where the protein is extended through substitution of the termination codon with an amino acid codon • For single amino acid in-frame deletion or insertions use at **supporting** • Do not use in combination with PVS1 or PP3	

PM5

_STR
_MOD
_SUP

Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before

- Used for missense variants where a **different** missense change has been reported at the same residue
- Consider variants both in the literature and from in-house databases
- Beware of changes that impact splicing rather than at the amino acid/protein level
- Beware that different amino acid changes can lead to different phenotypes
- Ignore the word 'novel'
- Can be upgraded to a **strong** level of evidence if there are multiple observations of different pathogenic variants at the same residue
- If the variant is classified as likely pathogenic and there is only one case reported then use at **supporting** level

PM6

_VSTR
_STR
_MOD
_SUP

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

- Use if identity is assumed but not confirmed
- If both maternity and paternity have been confirmed use **PS2**
- See PS2 for more details and guidance for using this code at differing strengths

PP1

_STR
_MOD
_SUP

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

- May be used as stronger evidence with increasing segregation data. The thresholds suggested by Jarvik and Browning (2016) should be used www.cell.com/ajhg/fulltext/S0002-9297(16)30098-2
- Consider the number of **meioses** (*m*), not number of informative individuals
  - The number of meioses from multiple families should be combined
  - Must consider: penetrance, age of onset, phenocopy rates and mode of inheritance
- For dominant disorders the probability that the observed variant-affected status data occurs by chance is $N = (1/2)^m$
- For recessive disorders:
  - If 2 affected siblings (proband plus sibling) share the same variants N = 1/4
  - If 3 affected siblings (proband plus two siblings) share the same variants N = 1/16
- For X-linked disorders:
  - When an affected male proband has either one affected brother with the variant, or one unaffected brother without the variant N = ½
- The gene must be associated with the disease presenting in your patient

PP2	_SUP

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

- Gives an idea about the number of observed/expected missense variants as a way of evaluating evidence of variable intolerance ("constraint") to missense variation across the gene
  - Genes or gene regions with significantly less missense variation than expected (i.e. more constrained) may represent genes where natural selection most aggressively removes variation
  - Values are based on ExAC or gnomAD data
- Z scores **≥3.09** (marked amber in ExAC) are significant but it is important to consider constraint for the **region** encompassing the variant, not just across the entire gene
  - For regional breakdown use DECIPHER or Table 2 from Samocha *et al* 2017 https://www.biorxiv.org/content/10.1101/148353v1
  - Missense constraint has not been evaluated for all genes
- Samocha *et al* 2017 explains the analysis

PP3	_SUP

Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc.)

- Base on Alamut conservation values along with *in silico* prediction tools SIFT, PolyPhen and AlignGVGD
  - No agreed rules, but consider using PP3 of if 2/3 tools predict deleterious effect, and using **BP4** if 2/3 tools predict benign effect and there is no conservation or 3/3 tools predict benign effect
  - AlignGVGD often gives benign C0 values due to gaps in alignment (can re-align if extra evidence needed)
- Use for variants outside the canonical splice acceptor (-1 and -2) and donor (+1 and +2) regions that are predicted to impact on splicing instead of **PVS1**
  - Use **PS3** instead if mRNA analysis demonstrates that the abnormal transcript is predicted to result in loss of protein expression
- Do not use in combination with PVS1 or PM4
- It is likely that a single meta-predictor tool (e.g. REVEL or GAVIN) will replace the use of multiple prediction tools for the assessment of missense variants

PP4	_STR _MOD _SUP
Patient's phenotype or family history is highly specific for a disease with a single genetic aetiology	
• Can be difficult to assess in the laboratory setting without complete clinical information and therefore MDT/clinical input may be necessary • The key consideration is the specificity of the phenotype • Does not need to be limited to diseases with a single genetic aetiology ○ **Can apply code if a patient has a rare combination of clinical features for which there are a limited number of known genetic aetiologies and all those genes (and relevant variant types) have been tested** • It is essential that (a) all the known genes associated with the disorder have been analysed using a highly sensitive method appropriate for the reported types of variants and (b) variants in these known genes explain the majority of cases with that clinical diagnosis • See Table 2 for examples of use at **supporting**, **moderate** or **strong**	

PP5	_SUP
Reputable source recently reports variant as pathogenic, but the evidence is not available to the laboratory to perform an independent evaluation	
• The ClinGen SVI group recommends that PP5 and BP6 criterion should **not** be used • **Exceptional cases** ○ For genes conferring susceptibility to certain cancers as majority of data reside in commercial companies and is not yet widely available	

BA1	_SA
Allele frequency is >5% in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium	
• Stand-alone evidence for benign classification	

BS1	_SA _STR

Allele frequency is greater than expected for disorder

- Tool available at http://cardiodb.org/allelefrequencyapp to determine if the allele frequency of the variant is greater than expected for the disorder (Whiffin *et al*. 2016)
    - In the absence of precise information about the disease prevalence and penetrance, use conservative settings (by selecting the highest likely prevalence and the lowest likely penetrance). For prevalence/incidence of rare disease see: www.orpha.net/orphacom/cahiers/docs/GB/Prevalence_of_rare_diseases_by_decreasing_prevalence_or_cases.pdf or www.orpha.net/orphacom/cahiers/docs/GB/Prevalence_of_rare_diseases_by_alphabetical_list.pdf
    - Determine if the variant frequency on gnomAD exceeds the maximum credible allele frequency
- Variants known to be pathogenic for dominant disorders should have allele frequencies in the general population below the disease incidence, and pathogenic variants for recessive disorders should have heterozygous frequencies consistent with their disease incidence
- For an autosomal dominant disorder with high penetrance it is acceptable to use this code as **stand-alone** evidence to classify a variant as likely benign
- Be cautious using this code based on a low number of alleles in gnomAD if the disease is late onset or has variable penetrance/expressivity

BS2	_STR

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

- Can use evidence from literature, gnomAD or in-house data but often more appropriate to use BS1
- Be cautious using this code based on a low numbers if the disease is late onset or has variable penetrance/expressivity
    - Remember you can only be sure that gnomAD doesn't contain data from individuals known to be affected by severe paediatric disease

BS3	_STR

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

- Functional studies for gene variants that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered the most well established and can include:
    - *In vitro* functional assays (e.g. reporter genes for transcription factors)
    - mRNA analysis for suspected splicing variants
- Evidence must be carefully assessed to determine the data quality, reliability and hence degree of confidence in the results
- *In silico* studies including protein modelling **cannot** be used

BS4	_STR

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

- The presence of **phenocopies** for common phenotypes (i.e. cancer, epilepsy) can mimic lack of segregation among affected individuals
- Be aware that families may have more than one pathogenic variant contributing to an autosomal dominant disorder, further confounding an apparent lack of segregation
- Biological family relationships should be confirmed to rule out adoption, non-paternity/maternity or sperm/egg donation etc.
- Be cautious using this code if variant is present in a seemingly unaffected relative
    - Especially if disease is late onset or has variable penetrance/expressivity
    - Careful clinical evaluation may be needed to rule out mild or sub-clinical symptoms

BP1	_SUP

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

- Only says 'primarily' so still consider using code if the vast majority of reported pathogenic variants are truncating even if there are also a few missense reported
- Can also be used in reverse…
    - 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)
    - But be sure what effect the variant has at the protein level

BP2	_SA _SUP

Observed *in trans* with a pathogenic variant for a fully penetrant dominant gene/disorder or observed *in cis* with a pathogenic variant in any inheritance pattern

- This requires testing of parents (or offspring) to determine phase
- Must be verified that the second variant is pathogenic or likely pathogenic
- Can be used if detected in another patient either in the literature or in the laboratory
- If two heterozygous variants of uncertain pathogenicity are identified in a recessive gene, then the determination of the cis versus trans nature does not necessarily provide additional information regarding their pathogenicity
- In certain well-developed autosomal dominant disease models, the detection of a pathogenic variant *in trans* may even be considered **stand-alone** evidence
    - This has been validated for use in assessing *CFTR* variants

BP3	_SUP
In-frame deletions/insertions in a repetitive region without a known function	
• Likely to be small in-frame deletions/insertions in repetitive regions, or regions that are not well conserved in evolution • If not present in a repeat region consider using **PM4** • Do not use in combination with **BP4**	

BP4	_SUP
Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.	

• Base on Alamut conservation values along with *in silico* prediction tools SIFT, PolyPhen and AlignGVGD
  ○ No agreed rules, but consider using BP4 of if 2/3 tools predict benign effect and there is no conservation or 3/3 tools predict benign effect
  ○ Only use only once in any evaluation of a variant i.e. do not count each algorithm independently
• Do not use if already using **BS3** if functional studies have shown no damaging effect on protein function or splicing
• Use in **combination with BP7** for synonymous variants affecting weakly conserved nucleotides which do not impact on splicing	

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

• There are exceptions:
  ○ Do not use for carriers of an unrelated pathogenic variant for a recessive disorder
  ○ Consider disorders in which having multiple variants can contribute to more severe disease. E.g. one pathogenic and one novel variant identified in a patient with a severe presentation of a dominant disease while a parent has mild disease
  ○ Consider disorders in which multigenic inheritance is known to occur, such as Bardet-Beidel syndrome, in which case the additional variant in the second locus may also be pathogenic but should be reported with caution
• Therefore this code is more useful for dominant disorders	

BP6	_SUP
Reputable source recently reports variant as benign, but the evidence is not available to the laboratory to perform an independent evaluation	
• The ClinGen SVI group recommends that PP5 and BP6 criterion should not be used	

BP7	_SUP
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	
• Use in combination with BP4 for synonymous variants affecting weakly conserved nucleotides which do not impact on splicing • Should still be cautious in assuming that a synonymous nucleotide change will have no effect • If computational evidence suggests a possible impact on splicing or there is raised suspicion for an impact (e.g. the variant occurs *in trans* with a known pathogenic variant in a gene for a recessive disorder), then the variant should be classified as uncertain significance until a functional evaluation can provide a more definitive assessment of impact or other evidence is provided to rule out a pathogenic role	