ACGS Best Practice Guidelines for Variant Classification in Rare Disease 2020

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1. Document Version History

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4.01	04/02/2020	Updated guidelines to replace 2019 version (previous versions 2017 and 2018)

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3. 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* 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 (for example 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). 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 highly-penetrant variants in patients with rare, monogenic diseases. Disease-specific

guidelines are being 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, mutational mechanism (e.g. haploinsufficiency, dominant negative), protein structure/function and the strength of the gene-disease relationship (Strande *et al* 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 prior probability that a patient has a disease-causing variant (or variant pair) in a specific gene 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. 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 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 that are thought to be of relevance according to the clinical presentation.

The level of detailed phenotype data provided with the laboratory referral depends upon the testing scenario and is optimally provided as a set of HPO (Human Phenotype Ontology) terms, however, it is recognised that certain phenotyping disciplines (e.g. neuroradiology, skeletal dysplasia) may have their own existing terminology or nosologies which may provide more detailed and appropriate description than HPO. For disorders where biochemical or other test results are critical for variant interpretation, this information may be provided via completion of a laboratory request form for the specific disorder. 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 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).

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://clinicalgenome.org/site/assets/files/3461/svi_proposal_for_de_novo_criteria_v1_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	Yes	*NIPBL*	Severe developmental delay; no	NOT USED

exome or genome (variants filtered by mode of inheritance)			other features of Cornelia de Lange	
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 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. ***See CanVIG guidance for use of PP4 for cancer predisposition gene variants.

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, multiple jaw keratocysts, palmar or plantar pits, non-specific radiological findings	N/A

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 syndrome	*ATRX*	N/A	Facial gestalt, severe intellectual disability in an affected male, consistent genital anomalies	HbH inclusion bodies
Supporting	ATRX syndrome	*ATRX*	N/A	Severe, intellectual disability in an affected male Family history	HbH inclusion bodies

				compatible with X-linked recessive inheritance	
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

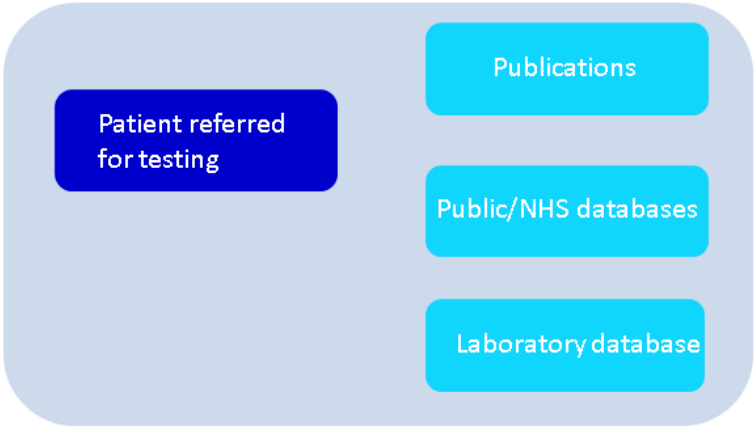
The ACMG/AMP variant classification guidelines may also be applied in interpreting sequence data from patients with common disease phenotypes where the purpose is to identify high penetrance genetic predisposition. Examples include familial breast or colorectal cancer, inherited cardiac conditions and 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.

5. 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).

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).

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



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). These updated combining criteria are summarised in Table 3.

Table 3: Updated combining criteria for classifying pathogenic or likely pathogenic variants from Tavtigian *et al* (2018). For original version from ACMG/AMP guidelines see Table 5 Richards *et al* (2015).

Classification	Combining rules
Pathogenic (a)	1 Very strong AND

	≥1 Strong OR ≥1 Moderate OR ≥2 Supporting
Pathogenic (b)	≥3 Strong
Pathogenic (c)	2 Strong AND ≥1 Moderate OR ≥2 Supporting
Pathogenic (d)	1 Strong AND ≥3 Moderate OR ≥2 Moderate AND ≥2 Supporting OR ≥1 Moderate AND ≥4 Supporting
Likely pathogenic (a)	≥2 Strong
Likely pathogenic (b)	1 Strong AND 1-2 Moderate OR ≥2 Supporting
Likely pathogenic (c)	≥3 Moderate OR 2 Moderate AND ≥2 Supporting OR 1 Moderate AND ≥4 Supporting

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 missense constraint evidence at the gene level (PP2) for a missense variant and classify a variant as of uncertain significance when all other evidence suggests that it is benign.

Table 4 (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.

Table 4: 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 or affecting alternative transcripts. 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).

We note that use of the PVS1 decision tree (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 only available for ~500 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.

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/3461/svi_proposal_for_de_novo_criteria_v1_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 outside the canonical ±1 or 2 splice sites through mRNA analysis from patient material or use of a minigene splicing assay. Note that PVS1 should not be used for non-canonical splice site variants. Evidence for non-canonical splice site variants should be assessed using PP3 (*in silico* only) or PS3 (if RNA studies have been carried out).

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. The use of appropriate control material should also be critically assessed, i.e. a functional assay should bear greater weight when results are equivalent or greater than known pathogenic variants tested in parallel, and when the output differs significantly than normal controls/non-pathogenic variants of the same context.

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 where 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 Genome Aggregation Database

It is important to check that the variant position is covered to sufficient read depth in gnomAD. 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.

PM2 can be used for autosomal or X-linked recessive disorders if there are no homozygotes/hemizygotes in gnomAD and the allele frequency is not greater than would be predicted for a benign variant with the disease prevalence, penetrance, genetic and allelic heterogeneity. 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 number of heterozygotes is consistent with the disease prevalence, penetrance, genetic and allelic heterogeneity. A very useful tool is available at http://cardiodb.org/allelefrequencyapp/ to support this process (Whiffin *et al* 2017). 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 genes such as *DNMT3A* and *ASXL1* during hematopoietic clonal expansion can occur with aging in healthy individuals. The age distribution and variant allele frequency can be checked in gnomAD to ascertain whether reported variants may be somatic.

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

A points-based system has been developed by the ClinGen Sequence Variant Interpretation group https://www.clinicalgenome.org/site/assets/files/3717/svi_proposal_for_pm3_criterion_-_version_1.pdf. PM3 can be used for the case being assessed if the patient is compound heterozygous and the other variant is (likely) pathogenic (without using PM3). 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 (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" i.e. 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.

PM6 - (Moderate) Assumed de novo, but without confirmation of paternity and maternity

When multiple patients have previously been reported, some with confirmation of parental relationships and others without, this evidence is reported as a single, combined criterion (PS2/PM6). See ClinGen SVI group points-based table as referenced in PS2.

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. 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 3 informative meioses, two families each with one informative meiosis or one family with 2 informative meiosis plus an additional unrelated case. Note that in the latter cases it would not be appropriate to also use PS4_Supporting. Co-segregation data can be used for autosomal dominant, autosomal recessive, X-linked and imprinted disorders.

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). 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.

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 MaxEnt 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 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 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).

BP4 – (Supporting) BP4 Multiple lines of computational evidence suggest no impact on gene or gene product

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.

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

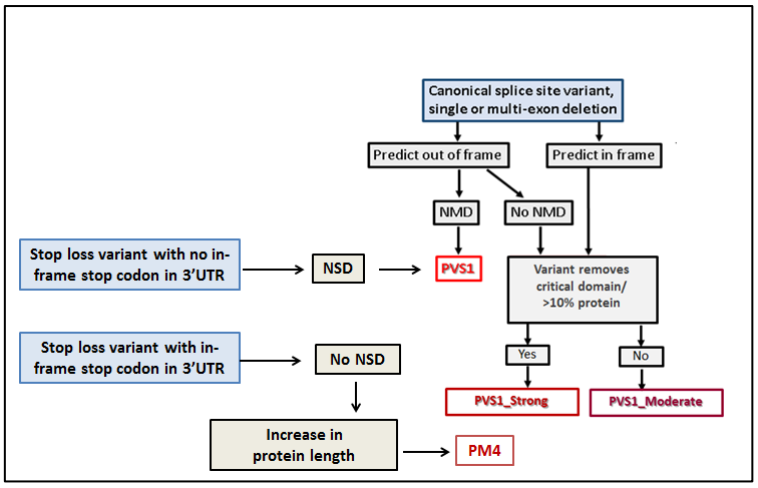
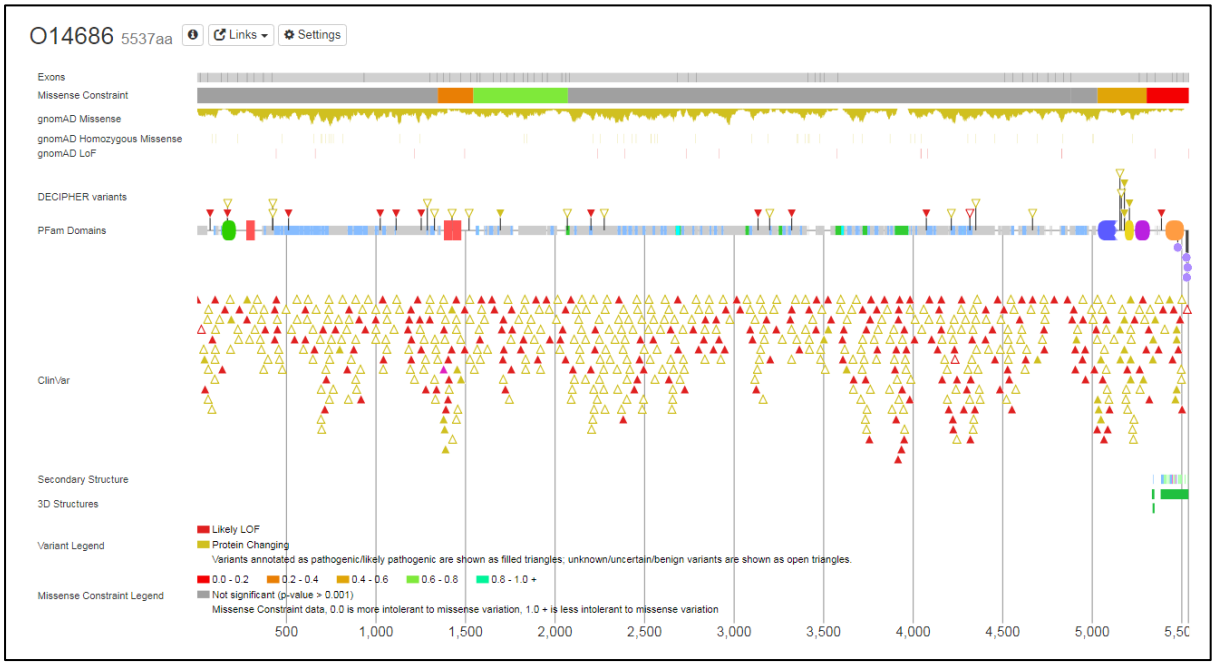


Figure 3: Example plot of *KMT2D* functional domains, ClinVar variants, proxy population/benign variants and missense constraint from DECIPHER (https://decipher.sanger.ac.uk/gene/KMT2D#overview/protein-info).



6. 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 (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 to that report should only describe those that are relevant, or have likely relevance, to the clinical question being addressed by the test. An example report is shown in Figure 4. 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 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 may change the classification.

For pathogenic or likely pathogenic variants, 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 (see Figure 4). We recommend that the evidence supporting the variant classification is included in an appendix to the report (see example in Figure 5). Variants classified as likely benign or benign should not be reported.

Figure 4: Example of a Genomic Laboratory Report

Lab website and contact details *Trust logo*

GENOMIC REPORT

Report to:
Consultant Paediatric Neurologist
Children's Hospital
Somewhere in England

Patient Name: Jean HELIX
Date of Birth: 02/11/2014
Sex: Female
NHS No.: 012 345 6789
Hospital number:
Family number:

Reason for testing
Diagnostic: to investigate the cause of Jean's muscle wasting and deafness.

Result summary
Genetic diagnosis of Brown-Vialetto-Van Laere syndrome type 2

Result
Jean is homozygous for a pathogenic *SLC52A2* missense variant (details below) previously reported by Johnson *et al* (2012 Brain 135:2875-82). Biallelic pathogenic *SLC52A2* variants cause autosomal recessive Brown-Vialetto-Van Laere syndrome type 2 (MIM614707).

Implications for treatment
High dose riboflavin supplementation has been reported to ameliorate the progression of this disorder (Foley *et al* 2014 Brain 137:44-56).

Recommended action
Testing of Jean's parents is recommended (via referral to the clinical genetics service) in order to confirm that both are heterozygous carriers of the missense variant.

Date issued: 09/11/2017 Authoriser: Clinical Scientist FRCPath

TECHNICAL INFORMATION

Variant details

Gene	Zygosity	Inheritance	HGVS description	Location: GRCh37 (hg19)	*Classification
SLC52A2	Homozygous	Not known	NM_024531.4:c.916G>A p.(Gly306Arg)	Chr8:g.145584068G>A	Pathogenic

Test methodology
Proband whole genome sequencing by the 100,000 Genomes Project with analysis of the PanelApp Paediatric motor neuronopathies (version 1.6) gene panel followed by in-house Sanger sequencing confirmation. Please note that the sensitivity of this test is limited by the types of detectable pathogenic variants, regions of low read depth coverage and incomplete ascertainment of disease-gene associations. Further information including read depth coverage is available on request. *Variants are classified using the ACMG/AMP guidelines (Richards et al 2015 PMID 25741868).

Patient phenotype
Neck muscle weakness; upper limb weakness; ataxia: sensorineural deafness.

Sample details
Project ID: 1234567
Laboratory No: 150001 Sample collected: 06/08/2016
Sample type: DNA from peripheral blood Sample received: 06/08/2016

Figure 5: Example of a Genomic Laboratory Report Appendix to describe evidence for the variant classification (note that the format of the evidence table may be configured according to local practice).

Lab website and contact details *Trust logo*

Appendix 1: Variant classification

Report to:
Consultant Paediatric Endocrinologist
Children's Hospital
Somewhere in England

Patient Name: John DOE
Date of Birth: 20/10/2013
Sex: Male
NHS No.: 212 345 6789
Hospital number:
Family number:

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 PMID 25741868 and ACGS https://www.acgs.uk.com/news/acgs-best-practice-guidelines-for-variant-classification-2019/)

- The p.Arg318 residue is located in the catalytic domain of the Glutamate Dehydrogenase (GDH) protein (PM1_Moderate).
- This 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 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).

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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 (see Figure 4) 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 (*see Figure 6 for sub-classifications of variants of uncertain significance). 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 X** OR **Genetic diagnosis of ("*Gene name")* - related disorder** OR **Confirms a genetic diagnosis of disorder X** (if there was clinical suspicion of this specific disorder)	Very high likelihood that the variant is causative of the disorder *>99% certainty that the variant is pathogenic*
Likely pathogenic	Yes	**Consistent with a genetic diagnosis of disorder X**	High likelihood that the variant is causative of the disorder *>90% certainty that the variant is pathogenic*
Uncertain significance *VUS where further testing or investigations could be considered as the results have the potential to change the classification to likely pathogenic*	Yes	**Inconclusive result – consider further action** Clearly describe in the report the action required that could change the classification to likely pathogenic	Further testing or investigations could be undertaken in order to re-classify the variant as likely pathogenic. Examples include: a) testing parents to determine whether *de novo* (PS2/PM6) b) mRNA analysis for variants predicted to affect normal splicing (PS3) c) testing affected

			relatives to show co-segregation (PP1) d) biochemical testing (PP4) e) trial of a treatment that is specific for the genetic aetiology (PP4)
Uncertain significance **hot/warm/tepid VUS where no further evidence can be obtained* ▪ 1 strong + 1 supporting OR ▪ 2 moderate + 1 supporting OR ▪ 1 moderate + 3 supporting OR ▪ 1 strong OR ▪ 2 moderate OR ▪ 1 moderate + 2 supporting OR ▪ 4 supporting OR ▪ 1 moderate + 1 supporting OR ▪ 3 supporting	Not usually	**A genetic cause for the patient's disorder has not been identified** 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 4.1.1).
Uncertain significance **cool/cold/ice cold VUS* ▪ 1 moderate OR ▪ 2 supporting OR ▪ 1 supporting OR ▪ No supporting evidence	No	**A genetic cause for the patient's disorder has not been identified** 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 4.1.1).
Likely benign	No	**A genetic cause for the patient's disorder has not been identified** 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 *>90% certainty that the variant is benign*
Benign	No	**A genetic cause for the patient's disorder has not been identified**	These variants are not relevant and potentially confusing

		State in the result text that the result does not exclude a genetic diagnosis	if included in the report *>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 disorder has not been identified** 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 disorder has not been identified** 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

Figure 6: Diagram to illustrate the different ways to describe variants of uncertain significance with differing levels of evidence in support of pathogenicity

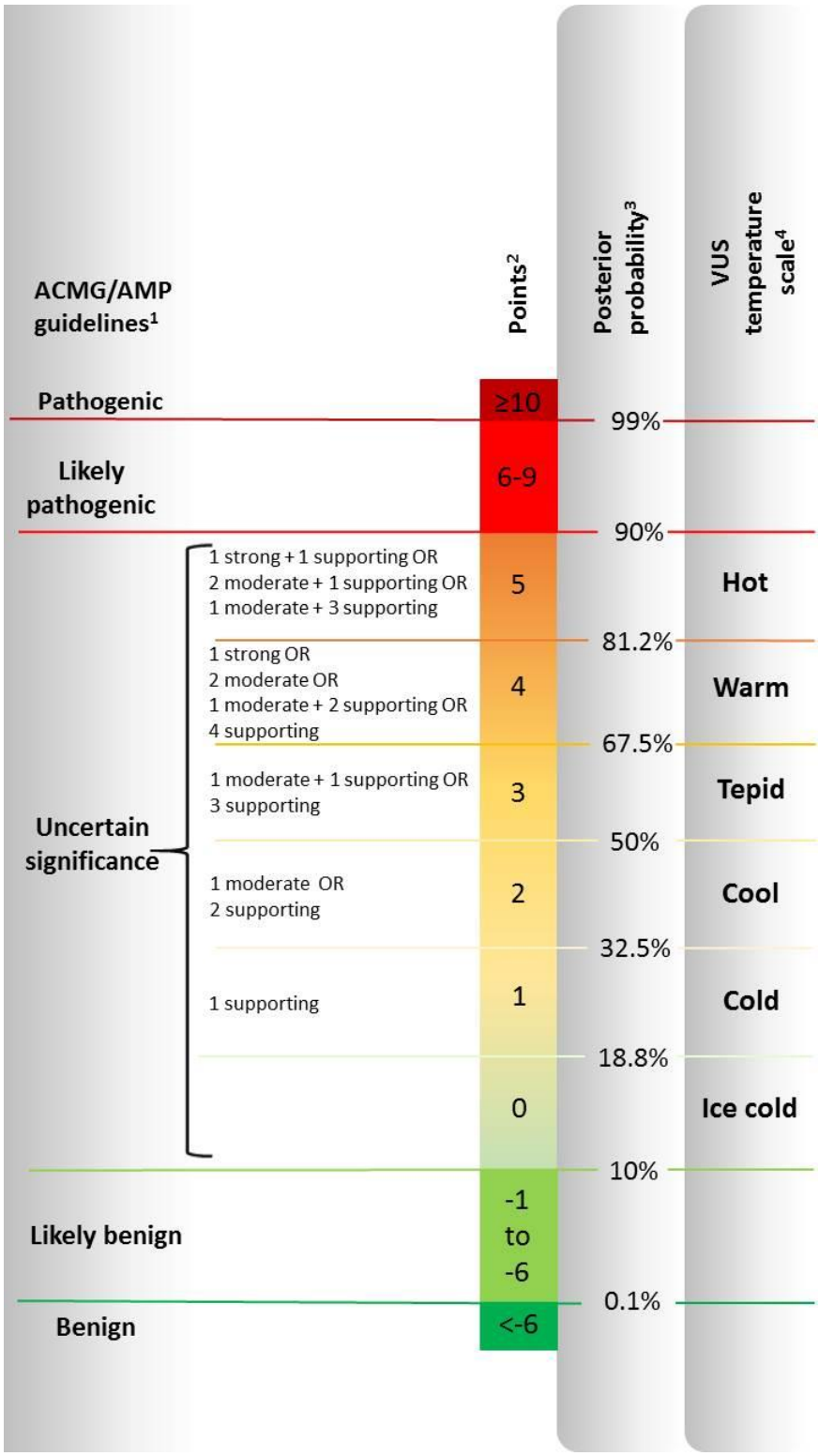

Table B: Additional recommendations for reporting genomic variants in recessive disorders

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** OR **Genetic diagnosis of ("*Gene name*")-related disorder** OR **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 *>99% certainty that each variant is pathogenic*
Recessive disorder with one pathogenic and one likely pathogenic variant identified and known to be in trans		**Consistent with a genetic diagnosis of disorder X**	High likelihood that the variants are causative of the disorder *>90% certainty that each variant is pathogenic*
Recessive disorder with two likely pathogenic variants identified and known to be in trans		**Consistent with a genetic diagnosis of disorder X**	High likelihood that the variants are causative of the disorder *>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**	High likelihood that the variants are causative of the disorder *>99% certainty that each variant is pathogenic*
Recessive disorder with one pathogenic and one likely pathogenic variant	Yes	**Possible genetic diagnosis of disorder X – parental testing required**	Likely that the variants are causative of the disorder

identified 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 *>90% certainty that each variant is pathogenic*
Recessive disorder with one (likely) pathogenic variant and a hot VUS	Yes	**Possible genetic diagnosis of disorder X – additional evidence required to confirm or refute 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 one (likely) pathogenic variant	Only appropriate in specific circumstances e.g. patient from a consanguineous family	**See section 6.1.3**	Either an incidental carrier finding or a second (likely) pathogenic variant has not been detected
Recessive disorder with one variant of uncertain significance identified	No	**A genetic cause for the patient's disorder has not been identified** 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 and potential risk of misinterpretation

6.1. 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). Richards *et al* (2015) also noted that *"some laboratories may choose to sub-classify VUSs, particularly for internal use"*. Figure 6 illustrates the different VUS categories according to the ACMG/AMP guidelines, using posterior probabilities estimated

from a Bayesian approach, on a points scale (derived from the Bayesian approach) and using a temperature gradient sub-classification which includes the (albeit unofficial) *"hot VUS"* description commonly used in the UK. The aim of Figure 6 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.

6.1.1. 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 *"hot/warm VUS*" 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) and parental relationships, if there are sufficient affected relatives (number of informative meiosis) available to show co-segregation (PP1), whether neuroimaging/muscle biopsy or a biochemical test could provide phenotype specificity evidence (PP4), 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 (PS3). If it is thought that 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: "**Inconclusive result – consider further action"** and that the further investigations or tests that might be undertaken are clearly detailed in the "Recommended action" section. This is particularly important for tests where discussion with the referring clinician is not feasible, for example high throughput tests that include the analysis of large gene panels in singletons where *de novo* variants are a common mechanism of disease. 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 is to prove that a variant with little supporting evidence in favour of pathogenicity is benign.

Exceptional circumstances

There may be situations in which an MDT discussion concludes that there is clinical utility in reporting a variant of uncertain significance. This would usually be at the *"warm/hot"* VUS level, 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 hot VUS. 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 confirm or refute this result".** The results section should include a statement to the effect that "additional evidence is required to confirm (or refute) this possible autosomal recessive diagnosis, for example identification of this variant in additional patients with a similar clinical presentation".

Where there is moderate evidence for pathogenicity, i.e. a *"tepid VUS",* the prior probability of pathogenicity is particularly important. For example if there are multiple affected individuals within the family, the specific gene (or biological pathway) was indicated by the referring clinician or the clinical presentation suggests a very high likelihood of a monogenic disorder.

Variants of uncertain significance where there is a lower level of supportive evidence include those for which there is either one moderate, two supporting or one supporting piece of evidence *"cool/cold/ice cold".* In most clinical settings and for most genes, these *"cool/cold/ice cold"* variants of uncertain significance are almost invariably unlikely to be disease-causing and should only be reported in exceptional circumstances. MDT discussion may be helpful for determining this. Most frequently these are novel missense variants (PM2 – absent from gnomAD) 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.

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 a "hot VUS". 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.

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

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

For single gene testing where the prior probability of a particular autosomal recessive disorder is high, the finding of a single monoallelic variant in 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, if we find a single monoallelic (likely) pathogenic variant in a gene associated with an autosomal recessive disorder 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 variants explaining the phenotype have been identified in another gene, then the decision as to whether to report a finding of incidental carrier status depends upon whether cascade testing would be offered for relatives and their partners. This is defined by clinical policies that take into consideration the disease prevalence, whether there are common variants that could be tested and whether there is known consanguinity. We do not recommend reporting of carrier status in these conditions as a default approach 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. Work is underway to develop policy for reporting of incidental findings.

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 another diagnostic testing method.

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

Variant classification is dependent upon current knowledge of reported gene-disease associations. An evidence-based framework for assessing these associations has been developed by the ClinGen group (Strande *et al* 2017). Variants identified within a candidate disease gene, 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 exome sequencing has not only identified many new disease genes not known 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.

6.2. 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. Bioinformatic analysis pipelines use different algorithms designed to prioritise rare variants according to criteria such as mode of inheritance, predicted effect on protein function and presence in disease databases such as HGMDpro. These pipelines then output lists of annotated genomic variants for manual review. The number of variants in a prioritised variant list is correlated with the number (and size) of the genes analysed and the specific filtering parameters that the pipeline uses. The majority of prioritised variants will be variants of uncertain significance since common variants classified as likely benign using the BA1 criterion are routinely filtered out. It is also important to understand that there are many reasons why a disease-causing variant (or variant pair) may not be included in the prioritised variant list. These include the specified filtering parameters of the pipeline (genes not in the gene panel used for analysis), variants 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. For this reason it is important to store sequence data in an accessible, secure way in order to permit future re-analysis within the laboratory. A systematic process for retrieving an individual patient's data is essential and is more readily achieved at the laboratory level than by storing lists of prioritised VUS obtained through a particular pipeline at one defined point in time in patient notes where there is no robust or reliable mechanism for review of these.

7. Reclassification of variants

Variant data and relevant associated information must be stored within the laboratory in a way that allows reclassification if required. National guidance is needed to define re-analysis of stored exome or genome sequence data for patients in whom no analysis is found during the initial testing process.

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. 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 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 and could be adopted UK-wide, 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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