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DNA variant classification–reconsidering "allele rarity" and "phenotype" criteria in ACMG/AMP guidelines

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ABSTRACT

Recent guidance suggested modified DNA variant pathogenicity assignments based on genome-wide allele rarity. Different *a priori* probabilities of pathogenicity operate where patients already have clinical diagnoses, and are found to have a very rare variant in a gene known to cause their disease, compared to predictive testing of a clinically unaffected individual. We tested new recommendations from the ClinGen Sequence Variant Interpretation Working Group for ClinVar-listed, loss-of-function variants meeting the very strong evidence of pathogenicity criterion [PVS1] in genes for 3 specific diseases where causal gene identification can modify clinical care of an individual- Von Willebrand disease, cystic fibrosis and hereditary haemorrhagic telangiectasia. Across these diseases, current rules leave 20/1,278 (1.6%) of loss-of-function variants as variants of uncertain significance (VUS that may not be reported to clinicians), and 207/1,278 (17.2%) as likely pathogenic. Applying the new ClinGen rule enabling PVS1 and the allele rarity criterion PM2 to delineate likely pathogenicity still left 8/1,278 (0.9%) as VUS (reflecting non-PVS1 calls by the submitters), and the majority of null alleles meeting PVS1 as merely likely pathogenic. We favour an approach whereby, for PVS1 variants in patients who personally meet the phenotypic PP4 criterion for a disease where casual variants are commonly family-specific, that PM2 is upgraded to permit a pathogenic call. Of 1,278 ClinVar-listed frameshift, nonsense and canonical splice site variants that met PVS1 in the 3 conditions, 16.0% (204/1,278) would be newly designated as pathogenic, avoiding misinterpretation outside of clinical genetics communities. We suggest further discussion around variant assessment across different clinical applications, potentially guided by PP4 alerts to distinguish personal versus family phenotypic history.

Assigning pathogenicity status to patient DNA sequence variants carries significant challenges. American College of Medical Genetics and Genomics and Association for Molecular Pathology (ACMG-AMP) guidelines were developed with a goal of improving standardisation (Richards et al., 2015). Establishing which criteria apply to a particular variant can be difficult, even for experienced interpreters. High discordance rates are reported, with inconsistent criterion application a common source of discordance (Amendola et al., 2020).

Depending on the healthcare system, variants of uncertain significance (VUS) are not always reported to clinicians, (Ellard et al., 2020) precluding any nuanced interpretation in the context of a patient already known to have disease. Avoiding a VUS label requires several criteria evidencing pathogenicity (Fig. 1). The "allele frequency" criterion is strong evidence of pathogenicity (PS4) if the variant is enriched in cases vs. controls in case-control studies, or is identified in multiple unrelated probands (Richards et al., 2015). Such unrelated probands do not exist for the rarest of alleles, or may not be accessible in scientific literature. In these settings, only the moderate allele frequency criterion PM2 ("absent from controls, or at extremely low frequency") is met. In 2020, ClinGen recommended downgrading PM2 from moderate to supporting evidence, to accommodate genome-wide evidence regarding allele rarity (ClinGen 2020).

Downgrading PM2 carries particular consequence for heritable monogenic disorders caused by null allele(s). Individual variants are rare due to the multitude of mechanisms that prevent production of a functional protein. Thus, PM2 is usually the strongest allele frequency

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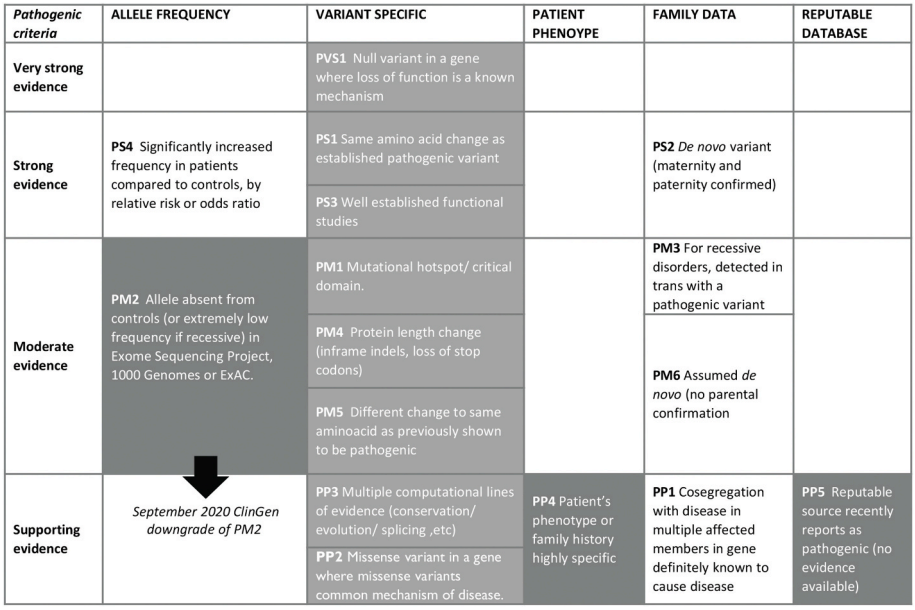

Fig. 1. Criteria applicable in a single patient setting: ACMG-AMP criteria that could apply to single patients with a rare disease where a firm clinical diagnosis is already available (white text; grey background, noting many variant criteria are mutually exclusive).

criterion available. Many strong and moderate evidence criteria demand wider family information which may not be available or resourced, particularly in adults. Furthermore, compared to four ACMG-AMP criteria concerning family data, there is only one (PP4) for disease phenotype, and this carries no greater weighting for clinically-diagnosed patients, than for unaffected family members (Table 1).

The current study explored the impact of applying a PM2 grade-change across three monogenic diseases caused by loss-of-function alleles (Von Willebrand Disease, cystic fibrosis and hereditary haemorrhagic telangiectasia). These three conditions were chosen because causal gene identification modifies patient care through enhanced surveillance where the full phenotype was not initially appreciated, or by enabling more effective, safer therapeutics (Rang et al., 2020; Connell et al., 2021; Buscarini et al., 2019).

First, we examined ClinVar-listed loss-of function variants meeting "very strong evidence of pathogenicity" (PVS1), listed in ClinVar (Landrum et al., 2020). To ensure PVS1 alleles did not fall back into the VUS category, ClinGen proposed a new rule permitting a PVS1-PM2 combination to yield a likely pathogenic classification (ClinGen 2020). In three genes (*VWF, ENG,* and *SMAD4*), examples were found where the original classification had failed to highlight PVS1, preventing application of the ClinGen-proposed new rule (Table 1A). These included early frameshift, nonsense, and canonical splice site variants, in addition to an 87bp insertion that introduced a stop codon in every reading frame, and a -1G>A splice variant in *SMAD4* where heterozygosity is a cancer susceptibility state demanding intense screening protocols from childhood. Table 1B lists 12 VUS variants that might, or might not, meet PVS1. In essence, where PVS1 is not recognised to be met, no ClinGen extra rule application could occur.

There is a much wider issue relating to greater use of "likely pathogenic" for very rare loss-of-function variants in genes where loss-of-function alleles cause a monogenic disease. Current ClinVar likely pathogenic listings include 137 variants in *CFTR* (44 early frameshift, 56 early nonsense and 37 splice site variants); 20 in *VWF* (8 early frameshift, 5 early nonsense and 7 consensus splice site variants); and 42 variants in HHT-causing genes *ENG, ACVRL1* and *SMAD4* (13 early frameshift, 5 early nonsense and 24 splice site variants). In these 5 genes alone, the ClinGen-proposed PM2 downgrade would relegate high proportions of the remaining 84.0% (1,074/1,278) of ClinVar listed pathogenic variants also to likely pathogenic. Conceptually, it is quite challenging to consider any uncertainty regarding these variants generating a null allele. That not all individuals with these variants develop disease refers to separate considerations (e.g. two alleles required for autosomal recessive diseases; superimposed penetrance etc.), and indicate misunderstanding: "pathogenic" and "likely pathogenic" classifications have no bearing on disease phenotype predictions. However, "likely pathogenic" conveys a degree of uncertainty of causality that may impede clinical care, either due to clinician or patient misunderstanding.

Furthermore, these classifications impede scientific knowledge.

Table 1
ClinVar VUS-listed variants potentially meeting PVS1:
Exemplar genes were selected where molecular diagnosis modifies management in circulatory and respiratory diseases: *VWF* (Von Willebrand disease); *CFTR* (cystic fibrosis); and *ENG*, *ACVRL1* and *SMAD4* (hereditary haemorrhagic telangiectasia, HHT). Briefly, using GRCh38(hg38) reference sequences for *ACVRL1*: NM_000020.3; *ENG*: NM_001114753.2; *SMAD4*: NM_005359.6; *CFTR*: NM_000492.4, and *VWF*: NM_000552.4, PVS1 was operationalised to include all variants affecting consensus (±1,±2) splice sites, and nonsense or frameshift variants likely to be subject to nonsense mediated decay, i.e. more than 55bp upstream of the final exon-exon boundary (Nagy and Maquat, 1998; Kurosaki et al., 2019). Variants meeting PVS1, but classified on ClinVar as Likely Pathogenic or a VUS, were evaluated with respect to both PVS1 status, and population allele frequency in the Genome Aggregation Consortium (gnomAD) databases comprising 213,158 exomes and genomes across v2.1.1 and v3.1 (Karczewski et al., 2020). As the selected diseases demonstrate variable penetrance or recessive inheritance, PM2 was considered to be met if the variant was absent, or displayed a very low population allele frequency (<0.00044). **A) PVS1 overlooked.** Variants were submitted by 5 different laboratories in North America and Europe. **B)** PVS1 may or may not be met. Variants were submitted by 9 different laboratories in North America, Europe and worldwide **a.** Note highlighted stop codons in all 3 reading frames. **b.** consensus splice site loss therefore NMD positional considerations do not apply. **c**. VUS designation unchanged for very rare frameshift and nonsense variants resulting in stop codons in final two exons that would not be subject to NMD, or where splicing consequence is uncertain.
d Consensus splice site duplication meets ACMG PVS1 criterion, but excluded because loss of function is uncertain (tested in Alamut). **e** Short, in-frame pan-exon deletion, and although loss of a functional CFTR domain is predicted (exon 15:transmembrane helix, exon 24:cytoplasmic C terminal domain), this is not relevant to the current PVS1-based evidence.

Disease Gene	Variant	Exon	PVS1 met?	gnomAD	PM2 met?	Current [5]	ClinGen PVS + PM2 rule	PM2 Upgrade
A) PVS1 overlooked in classifications								
VWF	c.792del (p.Cys265fs)	7 of 52	Yes, pre c.8198	Absent	Yes	VUS	VUS	Pathogenic
VWF	c.7483del (p.Leu2495fs)	44 of 52	Yes, pre c.8198	0.00002627	Yes	VUS	VUS	Pathogenic
VWF	c.8052C>A (p.Tyr2684*)	49 of 52	Yes, pre c.8198	Absent	Yes	VUS	VUS	Pathogenic
VWF	c.2442+1G>A	Splice variant	Yes **b**	Absent	Yes	VUS	VUS	Pathogenic
ENG	c.742_743insT (p.Asp248fs)	6 of 15	Yes, pre c.1797	Absent	Yes	VUS	VUS	Pathogenic
SMAD4	c.115delinsAA (p.Ala39fs)	2 of 13	Yes, pre c.1392	Absent	Yes	VUS	VUS	Pathogenic
SMAD4	c.1063_1064insGACCCTTCTGGAGGAGATCGCTTTTGTTTGGGTCAACTCTCCAATGTCCACAGGACAGAAGCCAT**TGA**GAGAGCAAGGTAT**TGA**T**TG**[**A**^{1064}…	10 of 13 **a**	Yes, pre c.1392	Absent	Yes	VUS	VUS	Pathogenic
SMAD4	c.1448-1G>A	Splice variant	Yes **b**	Absent	Yes	VUS	VUS	Pathogenic
B) PVS1 may or may not be met **c**								
VWF	c.6256+2dup	Splice variant	Uncertain **d**	Absent	Yes	VUS	VUS	VUS
CFTR	c.1680-2dup	Splice variant	Uncertain **d**	Absent	Yes	VUS	VUS	VUS
CFTR	del c.2491–1136_2619+2230 (del exon 15)	15 del	Uncertain **e**	Absent	Yes	VUS	VUS	VUS
CFTR	del c.3874-5122_3963+733 (del exon 24)	24 del	Uncertain **e**	Absent	Yes	VUS	VUS	VUS
CFTR	c.4387C>T (p.Gln1463*)	24 of 24	No, post c.4187	Absent	Yes	VUS	VUS	VUS
ACVRL1	c.1478del (p.Ser493fs)	10 of 10	No, post c.1322	0.00000398	*Yes*	VUS	VUS	VUS
ACVRL1	c.1507C>T (p.Gln503*)	10 of 10	No, post c.1322	Absent	Yes	VUS	VUS	VUS
SMAD4	c.1612G>T (p.Glu538*)	13 of 13	No, post c.1392	Absent	Yes	VUS	VUS	VUS
SMAD4	c.1645C>T (p.Gln549*)	13 of 13	No, post c.1392	Absent	Yes	VUS	VUS	VUS
SMAD4	c.1647del (p.Gln549fs)	13 of 13	No, post c.1392	Absent	Yes	VUS	VUS	VUS
SMAD4	c.249_249+6dup	Splice variant	Uncertain **d**	Absent	Yes	VUS	VUS	VUS
SMAD4	c.1139+2dup	Splice variant	Uncertain **d**	Absent	Yes	VUS	VUS	VUS

Table 2
Criteria and calls for 4 missense variants that defined 3 dimensional active sites in ENG protein. Original ACMG-AMP refers to designation using ACMG-AMP guidelines as in Richards et al. (2015) PM2 downgrade compared to ACMG, as in ClinGen 2020 (ClinGen,) PM2 upgrade compared to ACMG, as in Shovlin et al. (2020).

		Original ACMG-AMP	PM2 downgrade	PM2 upgrade
Cys[C]207Tyr,	**PM2**+PM1+PP2+ **PP4**	LP: 2 mod+2 supp	VUS: 1 mod+2 supp	LP: 1 str+1 mod+2 supp
Leu[L]300Pro,	**PM2**+PM1+**PP4**+PP5	LP: 2 mod+2 supp	VUS: 1 mod+3 supp	LP: 1 str+1 mod+2 supp
Leu[L]299Arg,	**PM2**+PM1+**PP4**	VUS: 2 mod+2 supp	VUS: 1 mod+3 supp	LP: 1 str+1 mod+1 supp
Lys[K]216Glu	**PM2**+PM1+**PP4**	VUS: 2 mod+2 supp	VUS: 1 mod+3 supp	LP: 1 str+1 mod+1 supp

Were the PM2 downgrade in use at the time of Shovlin et al., (2020) assignments, the identification of two hotspots for pathogenic variants, affecting non-contiguous amino acids, would not have been feasible (Table 2).

One way forward is wider recognition of the differing applications of gene testing in an already-diagnosed patient with altered *a priori* probabilities of pathogenicity for a variant identified in a known disease-causing gene, than a clinically unaffected/possibly affected family member who would currently receive the same PP4 criteria. The PP4 criterion could be weighted by phenotype specificity as currently applied to a very small number of diseases (eg 11 in Ellard et al., 2020). Our preference would be a general PP4 alert to a possible upgrade of PM2 in an already-diagnosed patient, according to disease subgroup-specifications regarding exactly what pattern of disease would deserve extra weighting. This would upgrade all VUSs listed in Table 1A, and allow the 1,278 ClinVar-listed PVS1 frameshift, nonsense and canonical splice site variants in the 6 genes currently listed as likely pathogenic, to be designated as pathogenic.

In conclusion, variant classification fields may have given insufficient consideration to gene testing of already-diagnosed patients. Clinical care for patients and families is substantially impacted by downgrades in assignments from likely pathogenic to VUS. The community embraced the rigour of the ACMG classifications but as shown by recent Position Statements that recognise reinterpretaion and recontact to be difficult issues in clinical and research care (David et al., 2019; Bombard et al., 2019; Carrieri et al., 2019), few centres routinely contact families affected by downgrades. The proposed more stringent ACMG/ClinVar criteria for allele frequency to prevent inappropriate calls in predictive settings impacts further on families and clinicians. Further exploration related to the clinical utility of genetic testing in mainstream testing seems warranted.

Declaration of Competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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