ClinGen Hearing Loss Variant Curation Expert Panel
ACMG/AMP Classification Rules for Hearing Loss

SUMMARY OF CLASSIFICATION CRITERIA

PATHOGENIC CRITERIA	
RULE	**RULE DESCRIPTION**
PVS1	Null variant in a gene with established LOF as a disease mechanism; see PVS1_Strong, PVS1_Moderate, PVS1_Supporting for reduced evidence applications
PVS1_Strong	See PVS1 flow chart for PVS1_Strong variants in gene where LOF is a known mechanism of disease
PVS1_Moderate	See PVS1 flowchart for PVS1_Moderate variants in gene where LOF is a known mechanism of disease
PVS1_Supporting	See PVS1 flowchart for PVS1_Supporting variants in gene where LOF is a known mechanism of disease
PS1	Same amino acid change as an established pathogenic variant; OR splice variants at same nucleotide and with similar impact prediction as previously reported pathogenic variant
PS2	2 points per tables 5a and 5b: Examples: 1 proven *de novo* occurrence; OR 2 assumed *de novo* occurrences
PS2_VeryStrong	4 points per tables 5a and 5b: Examples: 2 proven *de novo* occurrences; OR 1 proven + 1 assumed de novo occurrences; OR 4 assumed *de novo* occurrences
PS2_Moderate	1 point per tables 5a and 5b: Examples: 1 proven *de novo* occurrence (phenotype consistent but not specific to gene); OR 1 assumed *de novo* occurrence; OR 2 assumed *de novo* occurrences (phenotype/gene not specific)
PS2_Supporting	0.5 points per tables 5a and 5b: Example: 1 assumed *de novo* occurrence (phenotype/gene not specific)
PS3	Knock-in mouse model demonstrates the phenotype
PS3_Moderate	Validated functional studies show a deleterious effect (predefined list)
PS3_Supporting	Functional studies with limited validation show a deleterious effect
PS4	Fisher Exact or Chi-Squared analysis shows statistical increase in cases over controls, OR Autosomal dominant: ≥15 probands with variant, and variant meets PM2
PS4_ Moderate	Autosomal dominant: ≥6 probands with variant, and variant meets PM2
PS4_Supporting	Autosomal dominant: ≥2 probands with variant, and variant meets PM2
PM1	Mutational hot spot or well-studied functional domain without benign variation (*KCNQ4* pore-forming region*;* Gly residues in Gly-X-Y motifs of *COL4A3/4/5)*

PM2	Absent/Rare in population databases (absent or ≤0.00007 (0.007%) for autosomal recessive, ≤0.00002 (0.002%) for autosomal dominant)
PM2_Supporting	Low MAF in population databases (<0.0007 (0.07%) for autosomal recessive,
PM3	1 point awarded from tables 7a and 7b Example: Detected in trans with a pathogenic variant (recessive)
PM3_VeryStrong	4 points awarded from tables 7a and 7b Example: Detected in trans in ≥4 probands with a pathogenic variant (recessive)
PM3_Strong	2 points awarded from tables 7a and 7b Example: Detected in trans in 2 probands with a pathogenic variant (recessive)
PM3_Supporting	0.5 points awarded from tables 7a and 7b Examples: Two variants that meet PM2_Supporting detected in trans; OR a homozygous variant meeting PM2_Supporting
PM4	Protein length change due to an in-frame deletion or insertion that are not located in repetitive regions
PM5	Missense change at same codon as another pathogenic missense variant
PM5_Strong	Missense change at same codon as two different pathogenic missense variants
PM6	See PS2 above
PP1	Segregation in one affected relative for recessive and two affected relatives for dominant
PP1_Strong	Segregation in three affected relatives for recessive and five affected relatives for dominant
PP1_Moderate	Segregation in two affected relatives for recessive and 4 affected relatives for dominant
~~**PP2**~~	~~Missense in a gene with low rate of benign missense variants and pathogenic missense variants are common~~
PP3	REVEL score ≥0.7, or predicted impact to splicing using MaxEntScan
PP4	Patient's phenotype highly specific for gene or fully sequenced gene set (see specifications in Table 7)
~~**PP5**~~	~~Reputable source without shared data classified variant as pathogenic~~
BENIGN CRITERIA	
BA1	MAF of ≥0.005 (0.5%) for autosomal recessive; MAF of ≥0.001 (0.1%) for autosomal dominant
BS1	MAF of ≥0.003 (0.3%) for autosomal recessive; MAF of ≥0.0002 (0.02%) for autosomal dominant. Likely benign, provided there is no conflicting evidence.
BS1_ Supporting	MAF of ≥0.0007 (0.07%) for autosomal recessive. No BS1_Supporting criteria for autosomal dominant.
BS2	Observation of variant (biallelic with known pathogenic variant for recessive) in controls

	inconsistent with disease penetrance.
~~**BS3**~~	See BS3_Supporting
BS3_ Supporting	Functional study shows no deleterious effect (predefined list)
BS4	Non-segregation with disease
~~**BP1**~~	~~Missense variant in a gene where only truncating variants cause disease~~
BP2	Observed in trans with a dominant variant/observed in cis with a pathogenic variant (use with caution)
BP3	In-frame indels in repeat region without known function
BP4	Computational evidence suggests no impact; REVEL score ≤0.15 or no impact to splicing in MaxEntScan.
BP5	Variant in an autosomal dominant gene found in a patient with an alternate explanation
~~**BP6**~~	~~Reputable source without shared data classified variant as benign~~
BP7	Silent variant with no predicted impact to splicing

Strikethrough indicates rule was removed or not applicable. Abbreviations: MAF = minor allele frequency; Indels = insertion/deletions.

RULES FOR COMBINING PATHOGENIC CRITERIA

PATHOGENIC

1. 1 Very Strong AND
    a. ≥1 Strong OR
    b. ≥2 Moderate OR
    c. 1 Moderate and 1 Supporting OR
    d. ≥2 Supporting
2. ≥2 Strong OR
3. 1 Strong AND
    a. ≥3 Moderate OR
    b. 2 Moderate AND ≥2 Supporting OR
    c. 1 Moderate AND ≥4 Supporting

LIKELY PATHOGENIC

1. PVS1 AND PM2_Supporting[#] OR
2. 1 Very Strong AND 1 Moderate OR
3. 1 Strong AND 1-2 Moderate OR
4. 1 Strong AND ≥2 Supporting OR
5. ≥3 Moderate OR
6. 2 Moderate AND ≥2 Supporting OR
7. 1 Moderate AND ≥4 Supporting

[#] The addition of this rule is the only modification made from the original ACMG/AMP published guidelines for combining criteria.

RULES FOR COMBINING BENIGN CRITERIA

Benign

1. 1 Stand-Alone OR
2. ≥2 Strong

Likely Benign

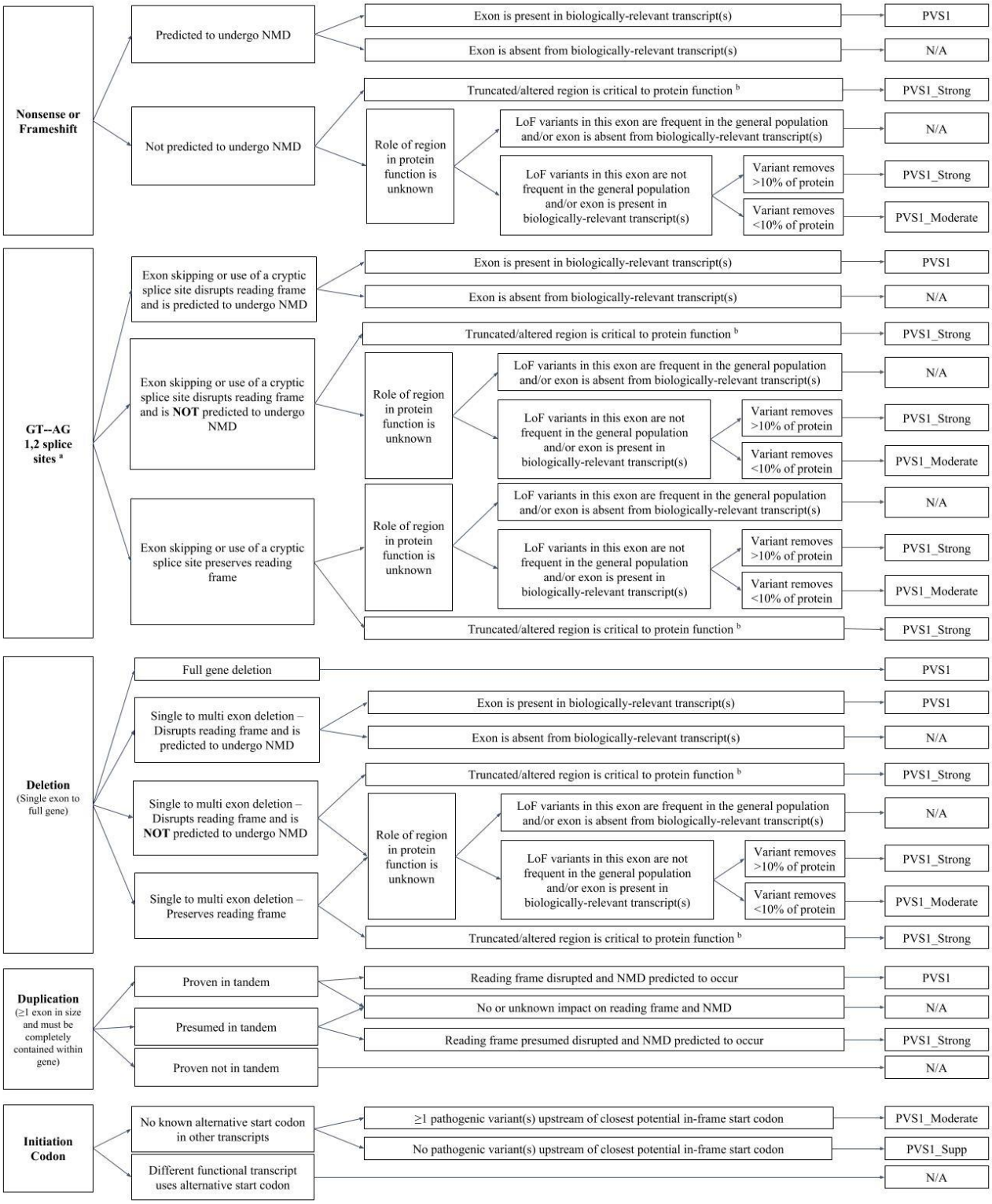
1. BS1 with no conflicting evidence[#]
2. 1 Strong and 1 Supporting OR
3. ≥2 Supporting

[#] The addition of this rule is the only modification made from the original ACMG/AMP published guidelines for combining criteria. The addition of this rule is consistent with the recommendations made by the Cardiomyopathy Expert Panel and the RASopathy Expert Panel.

EVIDENCE OF PATHOGENICITY

PVS1: Predicted null variant in a gene where LOF is a known mechanism of disease

- PVS1 should also be considered for the following genes with variants assessed in the Hearing Loss Variant Pilot: GJB2, CDH23, USH2A, SLC26A4, MYO6, MYO7A, TECTA, KCNQ4
- For other genes, LOF must be an established disease mechanism, and the gene/disease association must be Strong or Definitive clinical validity level as outlined in Strande et al. 2017 (PMID: 28552198)
- If above criteria is met, follow PVS1 flowchart as recommended by the SVI.



PS1: Same amino acid change as an established pathogenic variant

- Established variant must meet criteria for pathogenicity by the HL specifications
- Can also use PS1 for splice variants located in the splice consensus sequence, at the same nucleotide position as a previously reported pathogenic variant
    - Example: c.105+1G>C is known to be pathogenic, can use PS1 for c.105+1G>T
- No additional hearing loss specifications for missense variants. Follow recommendations as outlined in Richard 2015 and/or the Sequence Variant Interpretation working group within ClinGen.
- Caveat (from ACMG/AMP guidelines): Assess the possibility that the variant may act directly through the DNA change (e.g. through splicing disruption as assessed by at least computational analysis) instead of through the amino acid change)

PS2: De novo

- Follow recommendations as specified by the Sequence Variant Interpretation working group within ClinGen, as outlined below
    - Determine number of points per proband using table 1 below. Sum the total number of points for all probands, and determine the strength of the evidence by using table 2.

Table 1: Points awarded per de novo occurrence(s)

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

[†]Maximum allowable value of 1 may contribute to overall score

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

Supporting (PS2_Supporting or PM6_Supporting)	**Moderate** (PS2_Moderate or PM6)	**Strong** (PS2 or PM6_Strong)	**Very Strong** (PS2_VeryStrong or PM6_VeryStrong)
0.5 points	1.0 points	2.0 points	4.0 points

PS3: Well-established functional studies show a deleterious effect

- Recommend that functional evidence, except for a variant specific mouse model, is not used as strong evidence, due to the absence of well-established functional studies for hearing loss genes
- Guidance on functional evidence is as follows:
    - ***GJB2*: electrical coupling assays, dye transfer assays → PS3_Moderate**
        - Dye Transfer Assays: Expect results that compare the fluorescence of a variant-transfected cell to both a negative control (or H2O injected control) and a wildtype-transfected cell. PS3_Moderate

would be applied if the variant results in no dye transfer or significantly different dye transfer when compared to the wildtype.
        - Electrical Coupling Assays: Expect results comparing the current of the variant-transfected cells to both a negative control (i.e. H2O injected control) and a wildtype-transfected cell. PS3_Moderate would be applied if the variant results in significantly different current compared to the wildtype, and the current is comparable to background levels/negative control.
    - ***SLC26A4*: Radio isotope and fluorescence assays → PS3_Supporting**
        - Radio Isotope Assays: PS3_Supporting would be applied when cells transfected with mutant SLC26A4 show a statistically significant decreased efflux of iodide compared to wildtype pendrin.
        - Fluorescence Assays: PS3_Supporting would be applied when a cell transfected with the mutant SLC26A4 shows a statistically significant difference in fluorescence (ΔF_{max} %) compared to the wildtype protein, and when the fluorescence is not significantly different from that of an empty vector control.
    - ***COCH*: Localization, secretion, and dimerization studies performed using immunofluorescence and Western blotting techniques →PS3_Moderate**
        - Localization: PS3_Moderate would be applied if the mutant cochlin protein does not aggregate into extracellular deposits or in the perinuclear region, comparable to the localization of wildtype cochlin.
        - Secretion: PS3_Moderate would be applied if cochlin protein containing the variant does not show secretion from transfected cells, but aggregates in cell regions such as the ER, Golgi and nucleus or is degraded.
        - Dimerization: In a non-reducing environment, wildtype cochlin migrate quickly and appear smaller than in the reduced state because the structure is maintained by disulfide bonds. PS3_Moderate would be applied if the cochlin protein containing the variant forms more, or less, stable disulfide bonds when compared to the wildtype in non-reducing conditions.
- If not listed above, OK to use **PS3_Supporting** for other genes/functional analyses if
    - The assay has been validated by a known pathogenic and benign variant AND
    - There is plausible reason that the function the assay is testing relates to the phenotype AND
    - The assay conditions are likely to mimic the physiological environment.

PS4: Prevalence in affected individuals statistically increased over controls

- If a published case control study exists, use the data from the study, per ACMG/AMP guidelines
- Exclude cases with an alternate cause of disease from the below guidelines.
- **Autosomal dominant:**
    - In addition, if the variant meets PM2, the criterion may be applied with the strength noted based on the following proband count observations.

	Evidence	#
Proband Count (PS4) Autosomal Dominant Hearing Loss Only	**Strong**	**15**
	Moderate	**6**
	Supporting	**2**

- **Autosomal Recessive:**
    - If a published case control study does not exist, and the variant is reported at high frequency in both cases and controls, a Chi-squared or Fisher's Exact test can be performed to determine if the variant is statistically higher in cases than the general population. To use this, the gene must be definitively associated with hearing loss. Fisher's exact test is preferred if sample size allows. However, this should be done with

caution, since the general population databases are not a true control cohort, and could have individuals with hearing loss present. As such, this analysis can be used as evidence for pathogenicity, but should not be used as evidence against pathogenicity. The rule can be applied if the % of positive case alleles is higher than the % of positive alleles from the general population with a P value that is ≤0.05.

- Process:
  - Cases - From either publications or patient cohorts, determine the following, race-matching as closely as possible.
    - Number of positive case alleles
    - Number of negative case alleles
  - "Controls" - Using ExAC or gnomAD, determine the following, race-matching to cases as closely as possible.
    - Number of positive alleles
    - Number of negative alleles
  - Fill out a 2x2 contingency table in GraphPad QuickCalcs using the above data, using Chi-squared Test with Yates correlation a Two-tailed P value.

	Variant Positive Alleles	Variant Negative Alleles
Cases	#	#
General Population	#	#

PM1: Mutational hot spot or well-studied functional domain without benign variation

- KCNQ4 (NM_004700.3) gene - missense variants located within amino acids 271-292 can be awarded PM1. This region is the pore-forming intramembrane region where many variants that cause autosomal dominant hearing loss are located (Naito et al. 2013, PMID: 23717403; https://www.uniprot.org/uniprot/P56696). There are only two missense variants in this region in gnomAD, each with only single allele (http://gnomad.broadinstitute.org/; rs763326539: 1/33578 Latino chromosomes; rs55737429: 1/111720 European chromosomes).
- Collagen genes (*COL11A2*, *COL4A3*, *COL4A4*, and *COL4A5*): variants impacting glycine residues in the Gly-X-Y motifs

PM2 and PM2_Supporting - Absent/Rare in population databases

- Background: Rarity or absence in the general population is not robust evidence for pathogenicity, particularly for autosomal recessive disorders. However, the ACMG/AMP Guidelines were devised in such a way that absence or rarity were considered moderate evidence towards pathogenicity, and the framework requires multiple pieces of evidence to classify a variant as likely pathogenic or pathogenic.

	ACMG-AMP Criteria	MAF	Prevalence	Allelic Heterogeneity	Penetrance
AUTOSOMAL	BA1	≥0.005 (0.5%)	1/200[#]	7.2%[$]	100%
	BS1	≥0.003 (0.3%)	1/200	4.4%[&]	100%
	BS1_Supporting	≥0.0007 (0.07%)	1/200	1.0%*	100%
	PM2_Supporting	<0.0007 (0.07%)	Can apply PM2_Supporting if MAF is < BS1_Supporting (0.07%)		
	PM2	≤0.00007 (0.007%)	Can apply PM2_moderate if MAF is an order of magnitude below BS1_Supporting (ie ≤0.007%);		
AUTOSOMAL DOMINANT	BA1	≥0.001 (0.1%)	1/30[£]	5%[¥]	80%[β]
	BS1	≥0.0002 (0.02%)	1/150[π]	5%	80%
	PM2	≤0.00002 (0.002%)	Can apply PM2_Moderate if MAF is an order of magnitude below BS1 (ie ≤0.002%);		

[#] Congenital and childhood onset hearing loss, based on Morton and Nance, Lin 2012

[$] Rationale = Based most common variant (35delG) in the most common AR gene, 7% derived from LMM data

[&] Based 2nd most common variant (Val37Ile) in the most common AR gene, 4% derived from LMM data

* Based most common variant (2299delG) in the 2nd most common AR gene (USH2A), 1.2% derived from LMM data

[£] Prevalence derived: 1/15 x 50% - 1/15 = based on NHANES data from ages 40-49 (bilateral). 50% = based on % estimated to be due to genetic causes, in a pediatric population, therefore, likely an overestimate in adults

[¥] Allelic heterogeneity x genetic heterogeneity (25% x 20% = 5%), agreed upon by HL-EP. Literature search of ~5% allelic het was supported by Hildebrand 2011, Iwasa 2016, and Naito 2013.

[β] Voted upon by HL-EP

[π] Prevalence of HL x % estimated to be genetic (1/15 x 10%). HL-EP estimates that no more than 10% of hearing loss that occurs between the ages of 0-49 is genetic

Notes on MAF Thresholds:

- Some genes are associated to both autosomal recessive and autosomal dominant hearing loss, and therefore for these genes the AD MAFs should be used for PM2 and PM2_Supporting, since these are the more conservative thresholds
- For PM2 and PM2 Supporting, use actual frequencies in gnomAD, do not apply confidence interval or filtering allele frequency.
- For BA1, BS1, and BS1_Supporting, use filtering allele frequency in ExAC or 95% confidence interval, typically using http://cardiodb.org/allelefrequencyapp/

PM3: Detected in trans in several probands with a pathogenic variant (recessive):

- Use the below point system as recommended by the Sequence Variation Interpretation working group. Determine appropriate points for each proband by using table 1. Sum the total number of points for all probands, and determine what strength evidence should be applied by using table 2.
- Use caution if the variant is observed in an isolated population in multiple probands, especially if the same pathogenic variant is observed in trans. Consider downgrading strength in this scenario

Table 1: Default points for scoring variants that are observed in trans (PM3 rules)

Classification/zygosity of other variant	Points per proband	
	Known in trans	Phase unknown
Pathogenic/Likely pathogenic	1.0	0.5
Homozygous occurrence (Max points from homozygotes=1.0)	0.5	N/A
Rare uncertain significance variant on other allele, OR Homozygous occurrence due to consanguinity, (Max point= 0.5)	0.25	N/A

Table 2: Recommendation for determining the appropriate ACMG/AMP evidence strength level for in trans occurrence(s)

Supporting (PM3_Supporting)	Moderate (PM3)	Strong (PM3_Strong)	Very Strong (PM3_Very Strong)
0.5 points	1.0 points	2.0 points	4.0 points

PM4 - Protein length changing variants

- No changes. Follow recommendations as outlined in ACMG/AMP guidelines and/or Sequence Variant Interpretation working group.

PM5 - Missense change at same codon as another pathogenic missense variant

- **PM5_MODERATE**: No changes. Follow recommendations as outlined in ACMG/AMP guidelines and/or Sequence Variant Interpretation working group.
- **PM5_Strong**: Located at an amino acid residue with known pathogenic variation (at least 2 other variants at the same site meet pathogenic criteria for based on independent data)
- *Caveat:* Assess whether the variants in question could have an impact at the DNA level, such as through splicing impacts

PM6: De Novo Occurrence - SEE PS2 RECOMMENDATIONS ABOVE.

PP1 - Segregation evidence

- Follow general recommendations from ClinGen's Sequence Variant Interpretation working group as outlined below.
- For both autosomal dominant and autosomal recessive segregation counting, do not count probands as a segregation.
    - Affected segregations = # affected individuals in the family with the variant (dominant) or variants (recessive) - 1.
- **Dominant segregations:**
    - LOD scores are calculated a with the following equation:

$$Z\ (LOD\ score) = \log_{10}\left(\frac{1}{0.5^{Segregations}}\right)$$

- Only count affected individuals (minus proband) that are positive for the variant.

- **Autosomal recessive segregations:**
  - LOD scores are calculated a with the following equation:

$$Z\ (LOD\ score) = \log_{10}\left(\frac{1}{0.25^{\#\ affected\ segregations}\ x\ 0.75^{\#\ unaffected\ segregations}}\right)$$

  - The "0.25" and "0.75" numbers used in this equation represent the risk of being affected vs. unaffected in a classic AR disease model in which both parents are carriers
  - Affected segregations are defined as affected family members (typically siblings) who harbor the variant in question and a second variant on the remaining allele.
  - Unaffected segregations are defined as unaffected family members, typically siblings, who are at risk to inherit the two variants identified in the proband. These individuals should be either wild-type for both variants identified in the proband, or a heterozygous carrier for a single variant.
  - Unaffected, carrier parents DO NOT count as unaffected segregations
  - There may be scenarios where individuals other than siblings could be counted as segregations, such as in families where one parent is affected with the autosomal recessive disorder, in large families with multiple branches, or in consanguineous families.

	General Recommendations (Phenocopy not an issue)		
	Supporting	**Moderate**	**Strong**
Likelihood	4:1	16:1	32:1
LOD Score	0.6	1.2	1.5
Autosomal dominant threshold	2 affected segregations	4 affected segregations	5 affected segregations
Autosomal recessive threshold	See Table 2	See Table 2	See Table 2

		General Recommendations (Phenocopy not an issue)										
		Unaffected Segregations										
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Affected segregations	**0**	0	0.12	0.25	0.37	0.5	0.62	0.75	0.87	1	1.12	1.25
	1	0.6	0.73	0.85	0.98	1.1	1.23	1.35	1.48	1.6	1.73	1.85
	2	1.2	1.33	1.45	1.58	1.7	1.83	1.95	2.08	2.2	2.33	2.45
	3	1.81	1.93	2.06	2.18	2.31	2.43	2.56	2.68	2.81	2.93	3.06
	4	2.41	2.53	2.66	2.78	2.91	3.03	3.16	3.28	3.41	3.53	3.66
	5	3.01	3.14	3.26	3.39	3.51	3.63	3.76	3.88	4.01	4.13	4.26
	6	3.61	3.74	3.86	3.99	4.11	4.24	4.36	4.49	4.61	4.74	4.86
	7	4.21	4.34	4.46	4.59	4.71	4.84	4.96	5.09	5.21	5.34	5.46
	8	4.82	4.94	5.07	5.19	5.32	5.44	5.57	5.69	5.82	5.94	6.07
	9	5.42	5.54	5.67	5.79	5.92	6.04	6.17	6.29	6.42	6.54	6.67
	10	6.02	6.15	6.27	6.4	6.52	6.65	6.77	6.9	7.02	7.15	7.27

PP2: Missense in gene with low rate of benign missense variants and pathogenic missense variants are common

- Advise against using this rule because there are few such genes that this would apply to, particularly genes associated to autosomal recessive hearing loss.

PP3: Multiple lines of computational evidence support a deleterious effect on the gene/gene product

- Use REVEL and MAXENTSCAN,
    - For missense variants, award PP3 if REVEL score is ≥0.7
    - If splicing is predicted to be impacted, either creation of a cryptic splice site, or disruption of a native splice site, award PP3
- For splice variants (except for canonical -/+1 or 2), use MAXENTSCAN
    - For -/+ 1 or 2 splice variants, do not use PP3 if you are using PVS1

PP4: Patient phenotype and/or family history highly specific for gene

- The HL-EP applied this rule to HL syndromes if all causative genes have been sequenced and the detection rate at least doubles when the added clinical feature is present.
- See table below for applicable gene-disease phenotypes
- Advise against using PP4 for patients with nonsyndromic or apparently nonsyndromic hearing loss, given genetic heterogeneity

Gene	Syndrome	Phenotype	Detection rate in unselected HL	Detection rate with specified phenotype
SLC26A4	Pendred syndrome	Hearing loss with enlarged vestibular aqueduct (EVA) and/or Mondini malformation (incomplete partitioning type 2)	2.6% (Sloan-Heggen et al., 2016)	50% for a single mutation (Albert et al., 2006; Azaiez et al., 2007; Chattaraj et al., 2017; B. Y. Choi, Madeo et al., 2009; Pryor et al., 2005)
MYO7A, *USH1C, CDH23*, *PCDH15*, *USH1G*	Usher syndrome Type I	Moderately-severe to profound hearing loss and retinitis pigmentosa (onset typically in first decade), +/- vestibular dysfunction	4.3% (Sloan-Heggen et al., 2016)	78.7% for 2 mutations (Le Quesne Stabej et al., 2012)
USH2A, *ADGRV1* (GPR98), *WHRN* (DFNB31)	Usher syndrome Type II	Mild to severe hearing loss and retinitis pigmentosa (onset typically in first or second decade).	2.9% (Sloan-Heggen et al., 2016)	60.3% (Le Quesne Stabej et al., 2012)

CLRN1	Usher syndrome Type III	Progressive hearing loss with retinitis pigmentosa (variable onset) and vestibular dysfunction	<1% (Sloan-Heggen et al., 2016)	50% (2/4) for 2 mutations (Le Quesne Stabej et al., 2012)
WFS1	LF – SNHL	Low frequency autosomal dominant sensorineural hearing loss	0.4% (Sloan-Heggen et al., 2016)	28.5% (2/7 cases, LMM unpublished data)
EYA1, *SIX1*	Branchio-oto-renal syndrome	**3 major – or – 2 major + 2 minor – or – 1 major + a 1st degree relative meeting criteria** **MAJOR**: Branchial anomalies, hearing loss/deafness, preauricular pits, renal anomalies **MINOR:** External ear anomalies, middle ear anomalies, inner ear anomalies, preauricular tags, other (facial asymmetry, palate abnormalities) (Chang et al., 2004)	0.2% (Sloan-Heggen et al., 2016)	45% Testing must include deletion/duplication analysis (Smith, 1993)
OTOF, *DFNB59*	ANSD	Auditory neuropathy spectrum disorder	1% (Sloan-Heggen et al., 2016)	9-50% (Matsunaga et al., 2012; Rodriguez-Ballesteros et al., 2008; Varga et al., 2006)
MYH9	MYH9-related disorders	Congenital macrothrombocytopenia and platelet macrocytosis (Pecci et al., 2014) *NOTE: Patient must also be tested for DIAPH1 with no DIAPH1 variants identified*	<0.1% (Sloan-Heggen et al., 2016)	>90% (Pecci et al., 2014; Pecci et al., 2008; Savoia & Pecci, 1993)
DIAPH1	AD hearing loss with macro-thrombo-cytopenia	Macrothrombocytopenia (Neuhaus et al., 2017; Stritt et al., 2016) *NOTE: Patient must also be tested for MYH9 with no MYH9 variants identified* *All truncating variants associated to AD occur in exon 27. Truncating variants in other exons have been associated with AR microcephaly.*	Detection rate is unknown. Rare syndrome, prevalence <1/1000000 (DIAPH1-related sensorineural hearing loss-thrombocytope	Unknown

			niasyndrome, n.d.)	
PAX3, *SOX10*, *MITF*, *EDN3*, *EDNRB*	Waardenburg syndrome	**<u>Two or more of the following:</u>** 1. Congenital SNHL 2. Pigmentary disturbances of iris 1. Complete heterochromia (two eyes of different color); 2. Partial or segmental heterochromia 3. Hypoplastic blue eyes – characteristic brilliant blue in both eyes 3. Pigmentary disturbances of hair 1. White forelock from birth or in teens 2. Premature graying before age 20 4. A first or second degree relative with 2 or more of criteria 1-3 5. *Dystopia canthorum (PAX3 variants)* 6. *Hirschsprung disease (SOX10, EDN3, EDNRB)* Modified from (Liu, Newton, & Read, 1995)	Detection rate is unknown. Rare syndrome, prevalence of 9/100000 (Pingault et al. 2010; Pingault, 2015)	>75% for WS1 and WS3 30% for WS2 50% for WS4 (Pingault et al., 2010) Testing must include deletion/duplication analysis
POU3F4	X-linked recessive deafness	Hearing loss + Incomplete partitioning type III (IAC dilation, fistulous connection between basal turn of cochlea and the IAC, cochlear hypoplasia, stapes fixation)	Detection rate unknown. Prevalence is unknown. Inner ear abnormalities are pathognomonic	≥26.7% Testing must include deletion/duplication analysis including 5' promotor region.
GPSM2	Chudley- McCullough syndrome	Congenital severe to profound hearing loss with brain abnormalities including frontal polymicrogyria, grey matter heterotopia, cerebellar dysplasia, ventriculomegaly with small frontal horns, agenesis of the corpus callosum, arachnoid cysts (Doherty et al., 2012).	Detection rate unknown. Rare. Prevalence is 1 / 1 000 000	>90% (Diaz-Horta et al., 2012; Doherty et al., 2012)

KCNE1, *KCNQ1*	Jervell and Lange-Nielsen syndrome	Congenital sensorineural hearing loss (must be bilateral and severe to profound) and a reproducible prolonged QTc interval ≥ 480 msec *Notes:* - GeneReviews states most patients reported to date have QTc ≥500msec - Schwartz Score: ≥3.5 points = high probability of LQTS; QTc ≥ 480 msec (3 points) plus congenital deafness (0.5 points) - Upper limit of normal QTc is 440msec for males and 460msec for post-pubertal females. (Alders, Bikker, & Christiaans, 1993)	Detection rate unknown. Rare. Prevalence is 1-9 / 1 000 000 (Celano et al. 2009)	≥90% (Tranebjaerg, Samson, & Green, 1993)

Abbreviations: LF-SNHL: Low frequency sensorineural hearing loss. WS1 = Waardenburg syndrome, type 1; WS2 = Waardenburg syndrome, type 2; WS3 = Waardenburg syndrome, type 3; WS4 = Waardenburg syndrome, type 4.

PP5: Reputable source classifies variant as pathogenic

- Do not use. Not expected to have scenarios where classification is provided in a database without supporting evidence.

EVIDENCE OF BENIGN

Minor Allele Frequency Evidence: BA1, BS1, and BS1_Supporting

- Using a 95% confidence interval, the frequency thresholds outlined in the chart below were set.
- Some genes are associated to both autosomal recessive and autosomal dominant hearing loss, and therefore the MAF for autosomal recessive hearing loss should be used for BA1, BS1, and BS1_Supporting, since these are the more conservative thresholds.
- Please see a list of high frequency pathogenic variants that should not be classified as benign or likely benign based on their allele frequency.

	ACMG-AMP Criteria	MAF	Prevalence	Allelic Heterogeneity	Penetrance
AUTOSOMAL	BA1	≥0.005 (0.5%)	1/200[#]	7.2%[$]	100%
	BS1	≥0.003 (0.3%)	1/200	4.4%[&]	100%
	BS1_Supporting	≥0.0007 (0.07%)	1/200	1.0%*	100%
	PM2_Supporting	<0.0007 (0.07%)	Can apply PM2_Supporting if MAF is < BS1_Supporting (0.07%)		
	PM2	≤0.00007 (0.007%)	Can apply PM2_moderate if MAF is an order of magnitude below BS1_Supporting (ie ≤0.007%);		
AUTOSOMAL DOMINANT	BA1	≥0.001 (0.1%)	1/30[£]	5%[¥]	80%[β]
	BS1	≥0.0002 (0.02%)	1/150[π]	5%	80%
	PM2	≤0.00002 (0.002%)	Can apply PM2_Moderate if MAF is an order of magnitude below BS1 (ie ≤0.002%);		

[#] Congenital and childhood onset hearing loss, based on Morton and Nance, Lin 2012

[$] Rationale = Based most common variant (35delG) in the most common AR gene, 7% derived from LMM data

[&] Based 2nd most common variant (Val37Ile) in the most common AR gene, 4% derived from LMM data

* Based most common variant (2299delG) in the 2nd most common AR gene (USH2A), 1.2% derived from LMM data

[£] Prevalence derived: 1/15 x 50% - 1/15 = based on NHANES data from ages 40-49 (bilateral). 50% = based on % estimated to be due to genetic causes, in a pediatric population, therefore, likely an overestimate in adults

[¥] Allelic heterogeneity x genetic heterogeneity (25% x 20% = 5%), agreed upon by HL-EP. Literature search of ~5% allelic het was supported by Hildebrand 2011, Iwasa 2016, and Naito 2013.

[β] Voted upon by HL-EP

[π] Prevalence of HL x % estimated to be genetic (1/15 x 10%). HL-EP estimates that no more than 10% of hearing loss that occurs between the ages of 0-49 is genetic

Notes on MAF:

- Use the filtering allele frequency in ExAC until it is available in gnomAD. If the variant is present at high frequency in the Ashkenazi Jewish population in gnomAD, you can calculate the filtering allele frequency using a 95% confidence interval by selecting "Inverse AF" at http://cardiodb.org/allelefrequencyapp/

Variant exclusion list for which BA1 or BS1 does not apply:

Gene	Transcript	cDNA	Protein	ClinVar ID	Pathogenicity	MAF*
GJB2	NM_004004.5	c.35delG	p.Gly12Valfs*2	17004	Pathogenic	0.97% (European)
GJB2	NM_004004.5	c.235delC	p.Leu79Cysfs*3	17014	Pathogenic	0.64% (EA)
GJB2	NM_004004.5	c.167delT	p.Leu56Argfs*26	17010	Pathogenic	1.63% (AJ)
GJB2	NM_004004.5	c.-22-2A>C	p.?	375406	Uncertain Significance	0.45% (AJ)
GJB2	NM_004004.5	c.71G>A	p.Trp24*	17002	Pathogenic	0.45% (SA)
GJB2	NM_004004.5	c.34G>T	p.Gly12Cys	44740	Likely Pathogenic	0.38% (Latino)
GJB2	NM_004004.5	c.109G>A	p.Val37Ile	17023	Pathogenic	8.19% (EA)
GJB2	NM_004004.5	c.101T>C	p.Met34Thr	17000	Pathogenic	2.00% (EF)
SLC26A4	NM_000441.1	c.919-2A>G	p.?	4840	Pathogenic	0.48% (EA)
SLC26A4	NM_000441.1	c.349C>T	p.Leu117Phe	43555	Pathogenic	0.51% (AJ)

*The highest subpopulation frequency in the Genome Aggregation Database (gnomAD) is shown. EA: East Asian; AJ: Ashkenazi Jewish; SA: South Asian; EF: European (Finnish)

BS2 - Observation in controls inconsistent with disease penetrance

- Advise caution when using this rule, since most of hearing loss is autosomal recessive, and autosomal dominant hearing loss could display reduced penetrance or variable expression.
- However, if **biallelic** observations in controls are inconsistent with disease penetrance, this may be applicable.

BS3 - Well-established functional studies show NO deleterious effect

- Recommend that functional evidence is not used as strong evidence, due to the absence of well-established functional studies for hearing loss genes See BS3_Supporting below.

BS3_Supporting - Well-established functional studies show NO deleterious effect

- Recommend that functional evidence is not used as strong evidence, due to the absence of well-established functional studies for hearing loss genes
- Guidance on functional evidence at supporting level is as follows:
    - ***GJB2*: electrical coupling assays, dye transfer assays → BS3_Supporting**
        - Dye Transfer Assays: Expect results that compare the fluorescence of a variant-transfected cell to both a negative control (or H2O injected control) and a wildtype-transfected cell. BS2_Supporting can be applied if the variant results in dye transfer comparable to the wildtype.
        - Electrical Coupling Assays: Expect results comparing the current of the variant-transfected cells to both a negative control (or H2O injected control) and a wildtype-transfected cell. BS2_Supporting would be applied if the variant results in a current comparable to the wildtype.
    - ***SLC26A4*: Radio isotope and fluorescence assays → BS3_Supporting**
        - Radio Isotope Assays: BS3_Supporting would be applied if the variant results in iodide efflux levels comparable to the wildtype.
        - Fluorescence assay: BS3_Supporting would be applied if the variant results in fluorescence comparable to the wildtype
    - ***COCH*: Localization, secretion, and dimerization studies performed using immunofluorescence and Western blotting techniques → BS3_Supporting**
        - Localization: BS3_Supporting would be applied if the variant results in extracellular deposits comparable to the wildtype.
        - Secretion: BS3_Supporting would be applied if the variant results in secretion comparable to the wildtype.
        - Dimerization: In a non-reducing environment, wildtype cochlin migrate quickly and appear smaller than in the reduced state because the structure is maintained by disulfide bonds. BS3_Supporting would be applied if the variant results in molecular weight and size comparable to the wildtype.
- If not listed above, OK to use BS3_Supporting for other genes/functional analyses if
    - The assay has been validated by a known pathogenic and benign variant AND
    - There is plausible reason that the function the assay is testing relates to the phenotype AND
    - The assay conditions are likely to mimic the physiological environment.

BS4 - Non-segregation with disease

- Phenotype+/genotype-
    - Strong evidence for benign
    - Be cautious when using this as the possibility for phenocopy is high. The hearing loss phenotype should be consistent within the family to consider it a non-segregation, though intra-familial variability has been reported. Factors to consider are:
        - Age of onset (ie. congenital/early childhood vs. adult onset)
            - Hearing loss prevalence increases significantly with age. A congenital hearing loss in a child and a late onset hearing loss in a grandparent would not be a consistent phenotype.
        - Severity (ie - mild vs. profound)
            - Minor differences may exist among family members
            - Keep in mind that progression in older individuals may account for a discrepancy between individuals.

        - Sex -based differences (infertility, genes on X chromosomes)
    - Audiogram shape
        - May not be completely consistent among family members even with same etiology.
- Genotype+/phenotype-
    - Confounding variables to applying this rule: Age-related/sex-related penetrance, variable expressivity, etc.
    - If the gene is associated with later onset and individual with the non-segregation is beyond the expected age that the hearing loss would occur, consider applying BS4_Supporting
    - Recommend only using for fully penetrant genes (typically genes associated with AR hearing loss)
    - Must be confident that patient is truly unaffected and a hearing loss is not missed or subclinical. Be cautious if only phenotyping was newborn hearing screening. Diagnostic audiometric testing (auditory brainstem response (ABR) or audiogram should be required).
    - Any evidence for reduced penetrance, do not use BS4

BP1 - Missense in a gene where only truncating cause disease

- Not applicable. Do not use.

BP2 - Observed in trans with a dominant variant / observed in cis with a pathogenic variant

- Use with caution. For genes that are associated with both dominant and recessive hearing loss, consider whether an earlier onset/more severe phenotype could be present if variant is identified in trans with a dominant variant.

BP3 - In frame indels in repeat without known function

- No changes. Follow recommendations as outlined in Richard 2015 and/or ClinGen's Sequence Variant Interpretation working group.

BP4 - Multiple lines of computational evidence suggest no impact on gene / gene product

- Use REVEL, award BP4 if score is 0.15 or lower. Make sure to also check MAXENTSCAN to rule out the creation of a cryptic splice site.

BP5 - Found in a case with an alternate cause

- Autosomal recessive: Do not use. An individual could be carrier of pathogenic variant and have an alternate cause. Therefore, BP5 shouldn't be used as evidence for benign in this case.
- Autosomal dominant: Can use BP5 as outlined by Richards 2015.
    - Caveat: consider whether multiple pathogenic autosomal dominant variants could cause a more severe phenotype or whether multigenic inheritance is known to occur (example: Bardet-Biedl syndrome).

BP6 - Reputable source without shared data = benign

- Do not use this criterion. Not expected to have scenarios where classification is provided in a database without supporting evidence.

BP7 - Silent variant with non-predicted splice impact

- No changes. Follow recommendations as outlined in Richard 2015 and/or ClinGen's Sequence Variant Interpretation working group.

Summary of Gene-Specific rules for Genes included in Variant Pilot:

Gene	Disease, Inheritance	PVS1 Applicable	PM1: Mutational hot spot or well-studied functional domain	Functional Assays	Phenotype (PP4) Applicable
CDH23	Usher syndrome, AR	Yes	N/A	N/A	Yes
COCH	Nonsyndromic HL, AD	N/A	N/A	Localization, secretion, and dimerization studies performed using immunofluorescence and Western blotting techniques	N/A
GJB2	Nonsyndromic SNHL, AR	Yes	N/A	Electrical coupling assays, dye transfer assays	N/A
KCNQ4	Nonsyndromic SNHL, AD	Yes	amino acids 271-292	N/A	N/A
MYO6	Nonsyndromic SNHL, AD	Yes	N/A	N/A	N/A
MYO7A	Usher syndrome, AR	Yes	N/A	N/A	Yes
SLC26A4	Pendred syndrome, AR	Yes	N/A	Radio isotope and fluorescence assays	Yes
TECTA	Nonsyndromic SNHL, AD	N/A	N/A	N/A	N/A
	Nonsyndromic SNHL, AR	Yes	N/A	N/A	N/A
USH2A	Usher syndrome, AR	Yes	N/A	N/A	Yes

Abbreviations: AR= autosomal recessive; AD = autosomal dominant; N/A = not applicable