Expert Specification of the ACMG/AMP Variant Interpretation Guidelines for Genetic Hearing Loss

Andrea M. Oza[1, 2], Marina T. DiStefano[1,3], Sarah E. Hemphill[1], Brandon J. Cushman[1], Andrew R. Grant[1], Rebecca K. Siegert[1], Jun Shen[1,3,4], Alex Chapin[5], Nicole J. Boczek[6], Lisa A. Schimmenti[7], Jaclyn B. Murry[1], Linda Hasadsri[6], Kiyomitsu Nara[8], Margaret Kenna[2,3], Kevin T. Booth[9,10], Hela Azaiez[9], Andrew Griffith[11], Karen B. Avraham[12], Hannie Kremer[13], Heidi L. Rehm[1,3,14,15], Sami S. Amr[1,3,4], and Ahmad N. Abou Tayoun[16, 17,18] on behalf of the ClinGen Hearing Loss Clinical Domain Working Group

[1]Laboratory for Molecular Medicine, Partners Healthcare Personalized Medicine, Cambridge, MA, USA

[2] Dept. of Otolaryngology and Communication Enhancement, Boston Children's Hospital, Boston, MA, USA

[3]Harvard Medical School, Boston, MA, USA

[4]Department of Pathology, Brigham & Women's Hospital, Boston, MA, USA

[5]ARUP Laboratories, Salt Lake City, UT, USA

[6]Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

[7]Department of Otorhinolaryngology, Clinical Genomics and Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, USA

[8]Division of Hearing and Balance Research, National Institute of Sensory Organs, National Hospital Organization Tokyo Medical Center, Tokyo, Japan

[9]Molecular Otolaryngology and Renal Research Laboratories, Department of Otolaryngology, University of Iowa Hospital and Clinics, Iowa City, Iowa, USA

[10]The Interdisciplinary Graduate Program in Molecular Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

[11]Audiology Unit, National Institute on Deafness and Other Communication Disorders (NIDCD), NIH, Bethesda, Maryland

[12]Department of Human Molecular Genetics and Biochemistry, Sackler Faculty of Medicine and Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel.

[13]Department of Otorhinolaryngology and Department of Human Genetics, Donders Institute for Brain, Cognition and Behaviour, Radboud university medical center, Nijmegen, Netherlands

[14]Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA, USA

[15]The Broad Institute of MIT and Harvard, Cambridge, MA, USA

[16]The Children's Hospital of Philadelphia

[17]The University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA

[18]Current address: Al Jalila Children's Specialty Hospital, Dubai, UAE

CORRESPONDING AUTHOR: Ahmad N. Abou Tayoun, ahmadat@gmail.com, Al Jalila Children's Specialty Hospital, Oud Metha Road, Al Jaddaf - Dubai, United Arab Emirates

ABSTRACT (200 WORD LIMIT)

Due to the high genetic heterogeneity of hearing loss, current clinical testing includes sequencing large numbers of genes, which often yields a significant number of novel variants. Therefore, the standardization of variant interpretation is crucial to provide consistent and accurate diagnoses. The Hearing Loss Variant Curation Expert Panel was created within the Clinical Genome Resource to provide expert guidance for standardized genomic interpretation in the context of hearing loss. As one of its major tasks, our Expert Panel has adapted the American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) standards and guidelines for the interpretation of sequence variants in hearing loss genes. Here, we provide a comprehensive illustration of the newly specified ACMG/AMP

hearing loss rules. Three rules remained unchanged, four rules were removed, and the remaining twenty-one rules were specified. Of the specified rules, four had general recommendations, seven were gene/disease considerations, seven had strength-level specifications, and three rules had both gene/disease and strength-level specifications. These rules were further validated and refined using a pilot set of 51 variants assessed by curators. These hearing loss-specific ACMG/AMP rules will help standardize variant interpretation, ultimately leading to better care for individuals with hearing loss.

KEYWORDS (5-6 KEYWORDS): Variant interpretation, hearing loss, deafness, ClinGen, ACMG/AMP guidelines, genetic diagnosis

INTRODUCTION

Sensorineural hearing loss (SNHL) is the most common congenital sensory deficit, with an estimated prevalence of 2-3 cases per 1000 individuals (Morton & Nance, 2006). Approximately half of SNHL in children is due to genetic causes, with 70% being nonsyndromic and 30% syndromic. Although variation in the *GJB2* gene is the most common cause of nonsyndromic genetic SNHL in many populations, there is high genetic and allelic heterogeneity, with over 100 implicated genes (Hereditary Hearing Loss Homepage http://hereditaryhearingloss.org) (Abou Tayoun et al., 2016; Alford et al., 2014).

Given the substantial genetic contribution to SNHL, a clinical genetics evaluation, including comprehensive sequencing, is recommended as part of a standard of care diagnostic work-up. Results of genetic testing can inform clinical management, particularly if a genetic syndrome is identified before the onset of additional clinical manifestations (Alford et al., 2014). The interpretation of sequence variants is a critical component of an accurate genetic diagnosis, and discrepancies in variant interpretation and classification have been well-documented and can

have serious implications for patient care (Amendola et al., 2016; Booth et al., 2015; Booth, Kahrizi et al., 2018; Harrison et al., 2017). The large number of genes sequenced during hearing loss genetic testing routinely yield a large number of novel variants, exacerbating the interpretation challenge. Illustrating the extent of discrepancies in variant interpretation, as of 04/12/2018, 257 (8.1%) of the 3162 sequence variants in the 9 major hearing loss genes represented in our variant pilot (*USH2A, GJB2, SLC26A4, MYO7A, KCNQ4, TECTA, MYO6, COCH*, and *CDH23*) had conflicting interpretations in ClinVar.

In an effort to foster accurate interpretation and to reduce discrepancies across laboratories, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published recommendations and guidelines for the interpretation of sequence variants (Richards et al., 2015). The ACMG/AMP guidelines contain several types of evidence that are weighted and categorized. Although these guidelines were intended to be used universally for all Mendelian disorders, certain criteria require gene- or disease-specific knowledge, a lack of which has been shown to contribute to discrepancies in variant interpretation (Pepin et al., 2016). In response to these issues, the NIH-funded Clinical Genome Resource (ClinGen, http://www.clinicalgenome.org) was launched as a centralized resource to provide guidance and tools for defining the clinical validity of gene and variant contributions to disease. Specifically, the ClinGen Hearing Loss Clinical Domain Working Group (HLWG) was established in 2016 to form Expert Panels to evaluate gene-disease associations, as well as standardize variant interpretation in hereditary hearing loss and related syndromes.

Here, we document the work of the Hearing Loss Variant Curation Expert Panel, hereafter referred to as the HL-EP. We adapted the ACMG/AMP guidelines for variant interpretation in the context of hearing loss. These specified rules were developed using the most common genes and syndromes that contribute to genetic hearing loss (HL); with the intent that most

specifications could be used broadly across the many associated genes. These hearing loss-specific guidelines will be used in the future by the HL-EP to submit variant interpretations to ClinVar as an "expert panel" submitter, with the goal of resolving discrepancies, moving VUSs towards Benign or Pathogenic and increasing the confidence in HL variant classifications in ClinVar.

MATERIALS AND METHODS

Hearing Loss Clinical Domain Working Groups

As a ClinGen Clinical Domain Working Group (CDWG), the Hearing Loss CDWG aims to create a comprehensive, standardized knowledge base of genes and variants relevant to syndromic and nonsyndromic HL. Members were identified and recruited based on their expertise in hearing loss, and are representative of diverse institutions worldwide, spanning Asia, Australia, Europe, and North America. Members include otolaryngologists, clinical geneticists, molecular geneticists, ClinGen biocurators, clinical researchers, and genetic counselors from over 15 institutions. The Hearing Loss CDWG has so far formed two major efforts defined by ClinGen as a Gene Curation Expert Panel, and a Variant Curation Expert Panel (HL-EP).

Specifications of the ACMG/AMP Guidelines

A smaller task team utilized biweekly conference calls in addition to email correspondence to review, specify, and reach consensus for each of the rules within the ACMG/AMP guidelines. Typically, 15-20 members from the Laboratory for Molecular Medicine (LMM), Children's Hospital of Philadelphia (CHOP), ARUP Laboratories, Molecular Otolaryngology and Renal Research Laboratories (MORL), and Mayo Clinic participated on each call. Members with specific areas of expertise (e.g. Pendred syndrome) were also contacted for relevant topics.

The chairs, ClinGen biocurators, and coordinator of the HL-EP also utilized weekly conference calls for additional planning and preparation. For the larger CDWG, a quarterly conference call was used to present specifications finalized by the smaller group and to obtain final approval from all members. Upon specifying the ACMG/AMP rules, conference calls and email correspondence were used for the variant pilot using a set of 51 variants from nine common HL genes that represent the varied inheritance patterns, evidence types and phenotypic spectrum seen across hearing loss cohorts (see Variant Pilot methods below). The final set of specified rules is shown in **Table 1**.

Pathogenic allele frequency for recessive hearing loss

The frequencies of the most common pathogenic variants in HL were determined using a cohort of 3673 patients tested at the LMM. The three most common sequence variants were c.35delG, p.Val37Ile, and p.Met34Thr in *GJB2,* while the fourth variant was c.2299delG in the *USH2A* gene (**Table 2**). Test-based cohorts for the *GJB2* (n=1375 probands) or *USH2A* (n=1887 probands) genes were used to calculate the disease population allele frequency of each one of those variants.

The allele frequencies were confirmed using another HL cohort (n=2066) tested at the Molecular Otolaryngology and Renal Research Laboratories (MORL; Iowa City, IA). Although their frequencies were slightly different, the rankings of the four most common pathogenic variants was recapitulated in this cohort (**Table 2**). The frequency differences can be attributed to the fact that the MORL cohort excluded patients with positive findings upon *GJB2* prescreening. Therefore, the higher allele frequency (from the LMM) for each variant was conservatively used in calculating BA1 and BS1 for recessive HL. Our calculations were made under assumption of Hardy-Weinberg equilibrium and can be obtained using a recently published web application (https://www.cardiodb.org/allelefrequencyapp/) (Whiffin et al., 2017).

Performance analysis of functional studies

To assess the performance characteristics (sensitivity, specificity, positive predictive value, negative predictive value) of the functional assays in each of *GJB2*, *SLC26A4*, and *COCH*, the number of true and false variant calls were determined as follows. Variants classified in ClinVar and included in functional assays were reviewed for classification accuracy and then used for informing performance. Pathogenic and likely pathogenic variants that had abnormal assay readout, defined as statistically significant deviation from wild type variant(s), were labeled as true positives. Normal assay readouts for pathogenic or likely pathogenic variants were considered false negatives. Similarly, benign and likely benign variants with normal assay readouts (i.e. does not significantly deviate from wild type variants) were counted as true negatives. False positives were benign or likely benign variants with abnormal assay readouts. A summary of true and false variant calls by five functional assays in the above three genes is shown in **Supplementary Table 1**.

Variant Pilot

Following the specification of the ACMG/AMP guidelines, the rules were refined by interpreting a set of 51 variants in the *GJB2*, *SLC26A4*, *USH2A*, *MYO7A*, *CDH23*, *COCH*, *KCNQ4*, *MYO6,* and *TECTA* genes. Each variant was assessed independently by two variant curators, and the variant classification and rules applied were reviewed on a conference call to resolve discrepancies and reach consensus. Curators utilized ClinGen's Variant Curation Interface (https://curation.clinicalgenome.org/) to assess and document the applicable rules for each variant.

RESULTS

Summary of Specifications

The HL-EP recommended specifications for 21 ACMG/AMP rules (**Table1**). Four rules had general recommendations on the application of the rule (PS1, PP3, BS4, BP4, and BP5). Seven rules had gene or disease based specifications (PS3, PM1, PM2, PP4, BA1, BS4, BP2). Seven rules had strength-level specifications (PVS1, PS2, PM3, PM5, PM6, PP1, BS3). Three rules had both gene/disease based specifications and strength-level specifications (PS4, BS1, BS2). No changes were recommended for three rules (PM4, BP3, and BP7), whereas four rules were considered not applicable (PP2, PP5, BP1, and BP6).

Two changes to the ACMG/AMP recommendations were made on how to combine criteria to classify sequence variants. The first is that a likely pathogenic classification can be reached if a variant in a gene associated with autosomal recessive HL meets PVS1 and PM2_Supporting. The second change was that a variant can be classified as likely benign if a variant meets BS1 without valid conflicting evidence that would suggest a pathogenic role.

It should be noted that we do not discuss the technical challenges associated with sequencing some of HL genes, such as homology, GC-rich and/or repetitive regions, which may affect variant calling and interpretation. False positive variants in these regions can exist in population or disease databases and may result in erroneous classifications. Laboratories should carefully assess the technical quality of any variant before its interpretation. A full list of technically challenging regions in 109 HL genes was recently characterized (DiStefano et al., 2018).

Population data (BA1, BS1, PM2)

Recessive and dominant forms of HL were distinguished with respect to the interpretation of variant frequency data from the general population. This is mainly due to differences in prevalence, penetrance, and gene contribution. Evidence-based estimates of these attributes

are critical for establishing recessive and dominant allele frequency thresholds, at which a benign or pathogenic criterion of any strength level might be assigned for a given variant **Table 3**. For this work, the ExAC and gnomAD population databases were used, though other databases with a minimum of 2000 alleles are also sufficient. Caution should be used if the cutoffs specified below are applied on an uncharacterized population or a population with minimal representation in ExAC or gnomAD.

Recessive hearing loss. Based on a comprehensive literature search, a prevalence of 1 in 200 was used to specify the BA1 rule and to derive the BS1 and PM2 values for recessive HL. This was a highly conservative prevalence estimate from different ethnic groups and accounted for congenital and childhood onset HL (up to 19 years of age) with suspected genetic etiology (Lin, Niparko, & Ferrucci, 2011; Morton & Nance, 2006). Although HL prevalence increases with age, other contributing genetic (mostly dominant) and non-genetic (environmental, such as noise or drug related) factors may become more relevant in late-onset HL.

Penetrance was considered complete for recessive HL, based on the Expert Panel's experience, with the exception of two variants, *GJB2* p.Met34Thr and p.Val37Ile, which were treated as exceptions. To derive conservative genetic and allelic heterogeneity values, the allele frequency of the most common pathogenic variants using HL patient cohorts tested at the LMM and MORL were calculated. The same four pathogenic variants were identified as most common among the two cohorts (See **Methods** and **Table 2**). The higher frequency value for each variant was used to derive a conservative minor allele frequency (MAF) threshold for BA1, BS1, and BS1_Supporting.

BA1 and BS1. Using an allele frequency web app, (https://www.cardiodb.org/allelefrequencyapp/), the MAF threshold for BA1 was set at ≥0.5% for

autosomal recessive HL. This was calculated by using a prevalence of 1/200, complete penetrance (100%), and the allele frequency of the most common pathogenic variant found in cases (7.2% for c.35delG in *GJB2* - **Table 2**), Genetic heterogeneity was set to 1 given that the 7.2% allele frequency used was derived across all genetic causes of hearing loss. We then reviewed all reportedly pathogenic variants with frequencies near or higher than this threshold and developed a BA1 exclusion list which includes two low penetrance variants *GJB2* p.Met34Thr and p.Val37Ile as well as several founder mutations(**Supplementary Table 2**). Any variant exceeding 0.5% in any subpopulation, aside from variants on the exclusion list (**Supplementary Table 2**), would default to a benign classification. When implementing these allele frequency thresholds, a 95% confidence interval is used to determine the exact allele count for the cutoffs which allows accurate application to populations of varying size.

The MAF threshold for BS1 was set at ≥ 0.3% for recessive HL. This was calculated by using the allele frequency (4.4%) of the second most common pathogenic variant, p.Val37Ile in *GJB2* (**Table 2**), while maintaining the same prevalence, penetrance, genetic heterogeneity, and statistical correction used for the recessive BA1 derivation. Variants exceeding this frequency and having no other conflicting evidence for pathogenicity, meet the "Likely Benign" classification, provided that they are not the exclusion list in **Supplementary Table 2**.

The BA1 and BS1 cutoffs were validated using an independent approach, where an extensive curation of the literature was performed to estimate disease attributes (prevalence, gene contribution, pathogenic allele frequencies) of the most common causes of HL (*GJB2*, *SLC26A4*, *USH2A*, and *MYO7A*) across several ancestral groups globally (**Supplementary Methods** and **Supplementary Tables 3-6**). This approach validated the MAF thresholds determined above.

BS1_Supporting, *PM2_Supporting and PM2*. To enable broader use of population allele frequencies, we defined supporting strength levels of BS1 and PM2. BS1_Supporting was set at a MAF threshold of ≥0.07% using the same metrics as BA1 and BS1 (**Table 3**), but using a 1% allele frequency which is consistent with the most common pathogenic variant in the second most common recessive gene (c.2299delG in *USH2A* - **Table 2 - Table 2**) and above the frequency for most known pathogenic variants (Supplemental **Table 2**).

PM2 was set at ≤ 0.007%, which is an order of magnitude lower than the PM2_Supporting value (**Table 3**) and equivalent to an allele count of 14 in ExAC and 27 in gnomAD. Variants with frequencies between 0.07% and 0.007% can be awarded supporting evidence for pathogenicity (PM2_Supporting) (**Table 3**).

Dominant hearing loss. HL prevalence generally increases with age but with decreasing monogenetic contribution (Cunningham & Tucci, 2017). Most dominant HL forms are postlingual and therefore are more difficult to distinguish from genetic forms (Shearer, Hildebrand, & Smith, 1993). We chose prevalence in individuals aged 0-49 years to minimize those affected by age related HL. This age range is consistent with the age of onset of the major HL genes, including *TECTA* (Alloisio et al., 1999; Mustapha et al., 1999; Verhoeven et al., 1998), *DFNA5* (Bischoff et al., 2004; Booth, Azaiez et al., 2018; Van Laer et al., 1998), *COCH* (Nagy, Horvath, Trexler, Repassy, & Patthy, 2004; Robertson et al., 1998; Street et al., 2005), and *WFS1* (Eiberg et al., 2006; Fukuoka, Kanda, Ohta, & Usami, 2007; Hogewind et al., 2010). Based on an NHANES study (n=7490 patients), 1/15 individuals are expected to have HL by 49 years of age (Lin et al., 2011). For dominant BA1 derivation, we conservatively assumed that 50% of those individuals have a genetic cause, and calculated a prevalence of 1/30 (**Table 3**). This is a deliberate overestimation given that 50% is the estimated genetic contribution to congenital HL, which is

the highest contribution among all age groups, and is therefore expected to be lower in adults.

Due to a lack of published data on dominant HL penetrance, we used an estimated penetrance of 80%, based on the collective research and clinical experience of its members. The relative contributions of known genes to dominant forms of HL were also assessed in a cohort of 2000 patients tested at MORL. Four genes, *TECTA*, *WFS1*, *KCNQ4*, and *COL11A2*, were the most common accounting for 20%, 13%, 13%, and 7%, respectively, of all dominant pathogenic variants. Furthermore, among all nonsyndromic dominant genes tested at the LMM (n=3076 probands), *KCNQ4* contributed the most for a total of four pathogenic and likely pathogenic variants in one proband each. Therefore, the maximum allelic heterogeneity for a dominant HL gene is conservatively 25%. Based on these data, we safely estimated that no dominant variant could contribute more than 5% of all HL in the 0-49 year age range. This is based on the assumption that the maximum contribution of one gene would be 20% (*TECTA*) and the maximum contribution of any one variant to a gene's HL would be 25% (*KCNQ4*) (i.e. 20% x 25% = 5%). Our literature search also confirmed a maximum allelic heterogeneity of 5% in any given gene (Hildebrand et al., 2011; Iwasa, Nishio, & Usami, 2016; T. Naito et al., 2013).

Using the above conservative estimates, we set the dominant BA1 cutoff at ≥ 0.1% (**Table 3**). To derive the BS1 value, we used a less conservative estimate of dominant prevalence by assuming only 10% of all HL between ages 0-49 years is genetic. Therefore, a prevalence of 1/150 (1/15 x 10%) was used, while keeping all other values as in BA1, leading to a ≥ 0.02% BS1 cutoff. An allele frequency an order of magnitude less than this cutoff (i.e. ≤0.002%) was then considered sufficient to be awarded moderate evidence of pathogenicity (PM2). Using a 95% confidence interval, this is equivalent to 5 alleles in ExAC and 10 in gnomAD (**Table 3**).

It is important to note the phenotypic heterogeneity is exhibited by several of the HL genes. Genes known to cause both dominant and recessive forms of HL, such as *TECTA, COL11A2, GJB2* and *MYO7A* should be treated per the more conservative recessive rules for applying BA1 and BS1 rules since the frequency cutoffs for recessive inheritance are higher than those of dominant. This will help to avoid erroneously misclassifying variants as benign or likely benign. However, when considering pathogenicity evidence, the PM2 rule that matches the proposed form of HL (dominant vs recessive) should be applied.

X-linked forms of HL were not specifically addressed by the HL-EP. However, based on their relative rarity and the fact that most X-linked genes are not strictly recessive or dominant, with carrier females often displaying milder disease, the recessive thresholds for BA1, BS1, and BS1_Supporting can be applied for both X-linked recessive and X-linked dominant HL.

PS4, PS4_Moderate, PS4_Supporting: Prevalence in affected individuals statistically increased over controls

Published case-control studies demonstrating a statistically increased presence of a variant in affected individuals compared to race and ancestry-matched controls can be used as strong evidence towards pathogenicity, as outlined in the ACMG/AMP guidelines. For many variants, published case-control studies may not exist, but a case-control comparison to ancestry-matched controls in gnomAD can still provide critical evidence. This is particularly true for recessive variants with relatively high frequency in the general population. The following recommendations were made for variants in which a published case-control study does not exist.

A Chi-squared or Fisher's Exact test using a 2x2 contingency table can be performed to determine if the MAF of a variant is statistically higher in cases compared to the general

population. This analysis can be used as strong evidence if the MAF of the variant is significantly different (i.e. the p-value of the Chi-squared or Fisher's Exact test is ≤0.05) in cases than in the general population. It is important to note that to do this comparison, the case and population alleles should be adequately ancestry-matched, the total numbers of positive and negative alleles should be known for both the case and the population cohorts, and the relevant gene must be definitively or strongly associated with hearing loss per the ClinGen Gene Curation framework (Strande et al., 2017).

Large general population databases, such as ExAC or gnomAD, are not true control cohorts and likely contain individuals with HL, given that HL is not screened out. As such, absence of statistical significance should not be used as evidence against pathogenicity.

The ACMG/AMP guideline allows for the counting of probands as evidence for PS4 in the absence of case-control studies. For autosomal recessive HL, this is achieved through PM3 (see below). For autosomal dominant HL, we adopted the specifications previously published by the ClinGen Cardiomyopathy Expert Panel (CMP-EP) given that dominant HL has similar attributes to cardiomyopathy in terms of penetrance and phenocopies (Kelly et al., 2018). These specifications state that if a variant is absent or extremely rare in the general population (i.e. PM2 is met), identification of ≥2, ≥6, or ≥15 probands with the same variant would reach supporting (PS4_Supporting), moderate (PS4_Moderate), or strong (PS4) evidence, respectively. The rule PM2 must be met to use these PS4 specifications. As noted above, PM2 for autosomal dominant HL was set at ≤0.002%, which is slightly lower than the PM2 value of ≤0.004% set by the CMP-EP.

COMPUTATIONAL AND PREDICTIVE DATA

Loss of function (LOF) variants (PVS1, PVS1_Strong, PVS1_Moderate, PVS1_Supporting)

The HL-EP and the ClinGen Sequence Variant Interpretation Working Group (SVI) group jointly refined this rule to be used for all recessive and haploinsufficient genes across most diseases including HL. This refinement included a detailed guideline for interpreting the PVS1 criteria across all LOF variant types (nonsense, frameshift, canonical splice site variants (+/-1,2), deletions, duplications, and initiation codon variants) while accounting for variant, exon and transcript-specific considerations and the varied evidence weights. This guideline is available in an accompanying paper in this issue (Abou Tayoun et al., this issue) and will not be discussed further here.

Variants affecting the same amino acid residue (PS1, PM5, PM5_Strong)

The ACMG/AMP guideline considers the presence of a known pathogenic variant at the same amino acid residue as either strong or moderate evidence of pathogenicity. This PS1 rule is applied if the variant is a novel nucleotide change but results in the same amino acid change as the known pathogenic variant, and the PM5 rule is applied if the variant is a novel missense change at the same amino acid residue as a known pathogenic missense variant. It should be noted the ACMG/AMP guidelines recommend assessing whether the variants in question could have an impact at the DNA level, such as through splicing impacts, before applying PS1 or PM5. The HL-EP added a strength modification to the PM5 rule (PM5_Strong) so that a strong level of evidence can be applied if a novel missense variant is located at an amino acid residue with at least two other pathogenic variants at the same residue.

The HL-EP also specified that PS1 can be applied for DNA variants located at the same nucleotide in the splice consensus sequence as a known pathogenic variant. Based on the most conserved regions known to impact splicing, we defined the splice consensus sequence here as the first base and last three bases of the exon, the -12 through -1 positions in the intron, and the

+1 through +6 positions in the intron. The +/-1,2 positions were excluded, given that these positions should be evaluated using PVS1 rules. In addition, splice prediction algorithms must predict a similar or greater effect than the comparator known pathogenic variant.

Computational predictive tools (PP3, BP4, BP7)

Computational tools are commonly used to inform variant interpretation. For missense variants, the HL-EP recommends using REVEL, a computational tool that combines predictions from commonly used algorithms for missense variants (Ioannidis et al., 2016). REVEL was selected based on recently published work from members of ClinGen as well as evaluation within the HL-EP and the SVI (Ghosh, Oak, & Plon, 2017). Furthermore, we recommend specific REVEL scores are reached to apply supportive evidence of pathogenicity (PP3) or supportive evidence of benign (BP4). PP3 can be applied with a REVEL score of ≥0.7, given that 95% of benign variants are excluded at this score (Ioannidis et al., 2016). BP4 can be applied with a REVEL score of ≤0.15, given that 95% of pathogenic variants are excluded at this score (Ioannidis et al., 2016). Variants with a REVEL score between 0.15-0.7 will not receive either rule (PP3 or BP4).

The HL-EP recommends using MaxEntScan when assessing potential splicing impacts at the DNA level for missense, silent, and splice consensus sequence variants outside of the canonical ± 1 or 2 sites. For variants at the canonical splice sites, PP3 cannot be applied if PVS1 is used (Abou Tayoun *et al*, this issue).

Computational and predictive data rules with no changes (PM4, BP3, BP7)

The HL-EP did not further specify the rules pertaining to protein length changing variants (PM4, BP3). However, the PVS1 flowchart mentioned above should be carefully followed for large in-

frame deletions or insertions, particularly those affecting one or more exons. Silent variants with no predicted splicing impact can have BP7 applied, with no specified changes.

FUNCTIONAL DATA

Functional studies (PS3, PS3_Supporting, BS4_Supporting)

Based on systematic assessment of the literature, the HL-EP determined that no functional assays met a strong level of evidence for or against pathogenicity, except for a variant-specific knock-in mouse model. This is mainly due to the lack of well-established functional assays with enough data to assess their validity in predicting variant pathogenicity in most HL genes. Except for the functional assays in the *COCH*, *GJB2* and *SLC26A4* genes described below, a PS3_Supporting or BS3_Supporting can be appropriately used for evaluating variants in other genes not specified here if the assay is well-validated with positive and negative controls and the results are consistent with a known protein function.

GJB2. This gene, which encodes the gap junction protein, connexin 26, is the most common cause of genetic HL and has been well studied. Several research groups have measured the impact of genetic variants on its function primarily using electrical coupling or dye diffusion assays in *Xenopus* oocytes or mammalian cell systems (Bicego et al., 2006; Choung, Moon, & Park, 2002; D'Andrea et al., 2002; Lee, Derosa, & White, 2009; Mani et al., 2009). In both settings, a mutant and wildtype control cDNA are first transfected into separate oocytes or cell lines. In the electrical coupling assay, *GJB2* protein function is examined by altering the voltage in one cell and then measuring a current change in a neighboring cell through patch clamping or ion injection and dye sequestering (Ambrosi et al., 2013; Bruzzone et al., 2003; Haack et al., 2006; Mese, Londin, Mui, Brink, & White, 2004; Zhang et al., 2005). In the dye diffusion assay, movement of fluorescent dyes between neighboring cells is quantified as a measure of *GJB2*

protein function. Variants disrupting *GJB2* function are expected to significantly reduce electrical coupling or dye transfer compared to control cDNA.

We searched the literature for variants that were assessed by these two assays, and that were validly classified as pathogenic, likely pathogenic, benign, or likely benign. We then determined the performance characteristics for each assay as described in the methods section and shown in **Supplementary Table 7**. Since both assays demonstrated a high sensitivity and positive predictive value, the HL-EP recommends using PS3_Moderate for any novel *GJB2* variant resulting in a statistically significant decrease or absence of current or dye transfer compared to wild-type run under the same experimental conditions. On the other hand, given the limited number of benign variants used as negative controls (**Supplementary Table 1**), the HL-EP was less confident regarding the ability of either assay to accurately predict benign effects, and in such cases, using BS3_Supporting is more appropriate.

It should be noted that in dye diffusion assays, a negative control (e.g. non-transfected or water injected control) should not display dye diffusion. In electrical coupling assays, water injected controls should display negligible currents at all potentials.

SLC26A4. The *SLC26A4* gene encodes an anion transporter, and its function has been assayed by measuring transport of radioactive anion isotopes (e.g. iodide or chloride) in cells expressing normal or mutant protein subunits (Bizhanova, Chew, Khuon, & Kopp, 2011; B. Y. Choi, Stewart et al., 2009; Dossena, Bizhanova et al., 2011; Dossena, Vezzoli et al., 2006; Gillam et al., 2004; Ishihara et al., 2010; Palos et al., 2008; Reimold et al., 2011; Scott et al., 2000; Yuan et al., 2012). Alternatively, other groups have used halide-sensitive fluorescent probes to measure anion transport (Cirello et al., 2012; Dossena, Bizhanova et al., 2011; Dossena, Nofziger et al., 2011; Dossena, Rodighiero et al., 2006; Fugazzola et al., 2007; Jang

et al., 2014; Pera et al., 2008). An assay readout is considered abnormal if it is statistically different than the wild-type vector in the same experiment where a negative control (empty vector) shows absent immunofluorescent staining compared to the wild type. In both the radioactive and fluorescence based assays, several cell lines usually including *Xenopus* oocytes, mammalian, or human cells can be used to express the relevant *SLC26A4* gene variants. As with *GJB2*, we determined the performance characteristics of both assays by reviewing the results from valid pathogenic/likely pathogenic and benign/likely benign variants in ClinVar (**Supplementary Tables 1** and **7**). Again, there was a limited number of benign variants to assess the ability of both assays to predict benign effects (**Supplementary Table 1**). Furthermore, compared to *GJB2*, the two assays had lower positive predictive values (**Supplementary Table 7**). Therefore, we recommend using PS3_Supporting or BS3_Supporting if a pathogenic or a benign effect, respectively, was suggested by either assay.

COCH. This gene encodes for the Cochlin protein, which is normally secreted into the extracellular matrix of the inner ear (Fransen et al., 1999; Robertson et al., 2006; Robertson et al., 1998). Immunofluorescence and western blotting techniques have been used to assay cochlin secretion, localization, and post-translational modifications subsequent to expression in bacterial cells such as *E. coli* or mammalian cell lines (Bae et al., 2014; Cho et al., 2012; Grabski et al., 2003; Jung, Kim, Lee, Yang, & Choi, 2015). Compared to wild-type protein, abnormal patterns include absence of extracellular deposition (i.e. no secretion), intracellular aggregation (i.e. in the endoplasmic reticulum and Golgi), and stable dimerization due to inappropriate disulfide bond formation interfering with protein secretion and/or function (Cho et al., 2012; Nagy et al., 2004; Street et al., 2005; Yao, Py, Zhu, Bao, & Yuan, 2010). Based on the high sensitivity and positive predictive value (**Supplementary Tables 1 and 7**) of these protein assays, we recommend using PS3_Moderate if a variant exhibits an abnormal secretion, dimerization, or localization pattern compared to wild-type protein. On the other hand, due to a

dearth of benign variants tested by these assays, we recommend using only BS3_Supporting if a variant shows a comparable pattern to wild type protein using these protein assays. No criteria should be applied if multiple assay results do not agree.

Mutational hot-spots or functional domains (PM1)

The HL-EP identified one gene, *KCNQ4*, in the variant pilot set that harbored a functional domain for which PM1 can be applied. Missense variants located in the pore forming region (amino acids 270 - 297) of the *KCNQ4* gene are eligible for PM1, based on this region's extreme intolerance to variation in the general population and significant enrichment of pathogenic missense variants observed in individuals with dominant sensorineural HL (T. Naito et al., 2013).

However, a systematic review of mutational hotspots or functional domains for all genes associated with hearing loss was not performed by the HL-EP. We acknowledge that PM1 can likely be applied to other such regions, such as variants impacting glycine residues in the Gly-X-Y motifs of the collagen genes (*COL11A2*, *COL4A3*, *COL4A4*, and *COL4A5*), which are essential for the triple helical structure formation (Chakchouk et al., 2015; I. Naito, Kawai, Nomura, Sado, & Osawa, 1996; Savige et al., 2016; Tryggvason, Zhou, Hostikka, & Shows, 1993).

SEGREGATION DATA (PP1, PP1_Moderate, PP1_Strong, BS4)

Segregation with disease is used as evidence towards pathogenicity, and with an increasing number of segregations, stronger evidence can be applied. Given that segregation evidence is not specific to HL, the HL-EP worked in conjunction with the SVI to develop guidelines that further specify PP1. Three levels of evidence were recommended for both autosomal dominant and autosomal recessive segregations. Each strength level was based upon likelihood ratios of

4:1 (LOD 0.6), 16:1 (LOD 1.2), and 32:1 (LOD 1.5) to count as supporting, moderate, and strong evidence, respectively (**Table 4a**). For autosomal recessive segregations, unaffected individuals with equal probability of inheriting the variant(s) in question, typically siblings of a proband, can be taken into account, as shown in **Table 4b**.

Non-segregations can be used as strong benign evidence (BS4), however this should be done with caution. For family members that have HL, but are negative for the variant(s) in question (phenotype-positive, genotype-negative), there is a possibility for a phenocopy. Indications of a potential phenocopy could include differences in phenotype, such as HL age of onset, severity, and the audiogram shape.

For family members that are positive for the variant(s) in question, but have normal hearing (genotype-positive, phenotype-negative), confounding factors that must be taken into consideration include age-related penetrance and variable expression. It is recommended that BS4 is only applied for genotype-positive, phenotype-negative scenarios for genes expected to have full penetrance, including most autosomal recessive genes. Furthermore, there must be confidence that the family member is truly unaffected. Diagnostic audiometric data, including ear specific audiograms or an auditory brainstem response (ABR) should be obtained. If there is any evidence for reduced penetrance, variable expression, or an age of onset later than the age of the unaffected person, BS4 should not be applied.

DE NOVO OCCURRENCE (PS2, PS2_VeryStrong, PS2_Moderate, PS2_Supporting and PM6):

As defined by the 2015 ACMG guidelines, PM6 stands for "*de novo*, maternity and paternity not confirmed" and PS2 stands for "*de novo*, paternity and maternity confirmed". Approximately 80% of nonsyndromic HL is inherited in an autosomal recessive pattern with *de novo*

occurrences being rare (Alford et al., 2014). However, *de novo* occurrences have been reported, particularly for genes associated with autosomal dominant or X-linked HL (Alvarez et al., 2003; J. W. Choi et al., 2015; Moteki et al., 2015; Yuan et al., 2009). Given that *de novo* strength-level specifications are not specific to HL genes, the HL-EP worked in conjunction with the SVI and adapted the current recommendations from SVI which are posted on the ClinGen website (https://www.clinicalgenome.org/working-groups/sequence-variant-interpretation/). Different levels of strength are applied to *de novo* occurrences depending on how many are observed, whether the phenotype is specific, and whether maternity and paternity are confirmed (**Table 5a and 5b**).

ALLELIC DATA (PM3, BS2)

PM3, PM3_Strong, PM3_VeryStrong: In trans with a pathogenic variant (recessive disorders)

For recessive disorders, identifying a variant *in trans* with a pathogenic variant on the second allele is considered evidence towards pathogenicity. This was previously defined in the ACMG/AMP guidelines as moderate evidence (PM3) (Richards et al., 2015). However, if the variant under consideration is found in multiple probands with pathogenic variants on the other allele, the strength of that evidence should be considered greater. Therefore, in conjunction with the SVI, we developed a scoring system to account for the number of compound heterozygous and homozygous probands identified with a variant, as shown in **Table 6**. Each instance that a variant is found in a proband, either in homozygosity or compound heterozygosity with a second variant, is given a specified number of points, and the total points corresponds to the strength of PM3 that can be applied. Other scenarios, such as homozygous variants in consanguineous families, and variants of unknown phase, were also accounted for.

Furthermore, if a variant has been observed in many probands, each with a different pathogenic variant on the other allele, the likelihood that the variants are *in trans* is higher even if phasing has not been performed in every case, assuming the gene has been fully sequenced to detect all variants. In these cases, point values can be adjusted based on confidence of trans occurrence.

BP2: Observation in trans with a pathogenic variant for dominant disorders or observation of a variant in cis with a pathogenic variant

The identification of a variant *in cis* with a pathogenic variant is supportive evidence for a benign interpretation. However, caution is recommended for the application of this rule for a case with an *in trans* observation of a dominant pathogenic variant. Careful assessment should include whether an earlier onset or more severe phenotype is possible, which could be consistent with an in trans pathogenic variant.

PHENOTYPIC DATA (PP4 AND BP5)

The HL-EP developed recommendations for PP4, which, in the ACMG/AMP guideline, was intended as supporting evidence of pathogenicity when the patient's phenotype is highly specific for a single 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. The rationale for using a doubling is based on a Bayesian model of the ACMG/AMP guideline whereby any supporting piece of evidence is equivalent to a doubling of likelihood (Tavtigian et al., 2018). The specific clinical features in addition to HL that are allowed for application of PP4 are shown in **Table 7**.

The HL-EP further specified the BP5 rule, which the ACMG/AMP guidelines defined as supporting evidence of benign when a variant is identified in a case with an alternate cause. The

HL-EP recommends that BP5 is not invoked for variants in autosomal recessive genes, given that affected individuals are frequently identified as carriers of a pathogenic recessive variants in genes unrelated to the cause of their HL. For variants in autosomal dominant genes, the rule can be applied; however, caution should be taken if the observation is seen with a more severe phenotype than typical given that the presentation may be due to an additive effect of multiple dominant mutations (e.g. George et al., 2016).

REMOVED AND NOT APPLICABLE RULES

The HL-EP recommends that PP2 (missense variants in genes with low rate of benign missense variation) and BP1 (missense variants in genes where only LOF variants cause disease) are not used. These are not applicable to any known genes associated with HL. In addition, the HL-EP supports the SVI's decision to remove the two rules pertaining to variant classifications from reputable sources without evidence available (PP5 and BP6) based on the published rationale (Biesecker & Harrison, 2018).

COMBINING CRITERIA RULES

The HL-EP recommends following the rules for combining criteria as outlined in the ACMG/AMP guidelines, with the addition of two modifications.

First, the HL-EP specified that PVS1 and PM2_Supporting can be combined to reach a variant classification of likely pathogenic for variants in autosomal recessive genes. The ACMG/AMP criteria require combining at least one moderate or two supporting criteria with a very strong criteria to reach a likely pathogenic classification. This was decided given that the HL-EP adopted the stringent criteria for evaluating predicted LOF variants as described above under PVS1 such that we feel that rare variants meeting at least PM2_Supporting have at least a 90% chance of being pathogenic.

The second addition that the HL-EP added to the rules for combining criteria was that variants meeting BS1 with no conflicting evidence can be classified as Likely Benign. This is consistent with approaches taken by the RASopathy and Cardiomyopathy Expert Panels (Gelb et al., 2018; Kelly et al., 2018).

Finally, per the new PVS1 rule recommendation (Abou Tayoun et al., this issue), we recommend against combining PVS1 with PP3 for the canonical ±1 or 2 splice bases given that PP3 is essentially applying predictive assumptions already built into PVS1.

VARIANT PILOT OF THE HEARING LOSS SPECIFIED RULES

A set of 51 variants in the *GJB2*, *MYO7A*, *USH2A*, *CDH23*, *MYO6*, *KCNQ4*, *COCH*, *TECTA*, and *SLC26A4* genes were selected to test and refine the above specifications set by the HL-EP. These variants were either present in ClinVar, present on the exclusion list for BA1 and BS1, or identified via literature search. All the major rule types supporting pathogenicity were tested, except for the rules specifying *de novo* occurrences which are extremely rare within HL (**Figure 2a**). Dual curation was performed, and then consensus was reached following discussion for all classifications and their supporting rules among the HL-EP. In addition, several rules, including PP1, PS4, PP3, BP4, and BP1_Supporting, were iteratively refined based on this variant pilot. A summary of the genes, phenotype, associated inheritance pattern, and gene-specific rules are shown in **Table 8**.

The list of the variants, their classifications and supporting rules are shown in **Supplementary Table 8**.

DISCUSSION

Specifying the ACMG/AMP guidelines for HL posed unique challenges, which arose from high prevalence, high genetic heterogeneity, and multiple types of inheritance patterns. While published specifications exist for cardiomyopathy and RASopathy, these specifications focus on autosomal dominant disorders that typically have pathogenic missense variants as the causal variants. In contrast, HL is frequently autosomal recessive with LOF as the primary disease mechanism, and therefore, the HL-EP specifications addressed and utilized rules that have not been previously refined for more quantitative and nuanced use, including PM3 and PVS1. These rules were some of the most commonly used rules in the variant pilot and are likely to be frequent among all recessive disorders. As such, these specifications could be applied or serve as a groundwork for other autosomal recessive disorders that rely heavily on biallelic proband observations and predicted LOF variants.

Furthermore, a new combining rule for addressing novel LOF variants was created to address "under-classifying" variants that the HL-EP felt should be classified as likely pathogenic. By adding a rule whereby PM2_Supporting and PVS1 can be combined to reach a likely pathogenic variant classification, the HL-EP allowed convincing LOF variants with MAFs consistent with pathogenicity to be classified as likely pathogenic while remaining conservative for other potential rule combinations.

The HL-EP expects that these specifications will evolve as they are utilized by diagnostic laboratories, and as more information on the genetic basis of HL is identified, such as additional autosomal dominant loci, adult-onset HL, and founder mutations in under-studied cohorts. Furthermore, ongoing general refinements to the ACMG/AMP guidelines may be made by the ClinGen SVI which will need to be addressed.

The HL-EP is in the process of submitting for full Expert Panel status for its work on variant interpretation, with the goal of submitting expert variant classifications to ClinVar. These classifications will resolve discrepancies, move VUSs towards Benign or Pathogenic and increase the review status of hearing loss variant classifications present in ClinVar.

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FIGURE LEGENDS

Figure 1. Population data criteria for autosomal recessive and autosomal dominant hearing loss. Minor allele frequencies are represented in decimals above each bar. Abbreviations:PM2_P = PM2_Supporting, BS1_P = BS1_Supporting.

Figure 2. Classifications of 51 pilot variants. A. The frequency with which ACMG rules were applied during variant pilot. Rules applied with a modified strength are denoted by the rule followed by _P for Supporting, _M for Moderate, _S for Strong, and _VS for Very Strong.
B. Final variant classifications of 51 pilot variants compared to initial classifications in ClinVar. The total height of each bar represents the total number of variants given each classification along the x-axis by the HL-EP. The colored segments of each bar represent variants whose ClinVar classification differs from the HL-EP classification.
C. Distribution of pilot variants (n=51) by gene.

Autosomal recessive

PM2	PM2_P	BS1_P	BS1	BA1
0.00007		0.003		0.005

Autosomal dominant

PM2	BS1	BA1
0.00002	0.0002	0.001
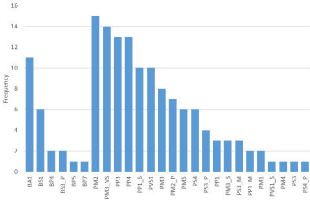
Final Expert Panel Classifications
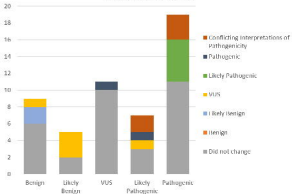
Table 1: ACMG Criteria with HL-EP Specifications

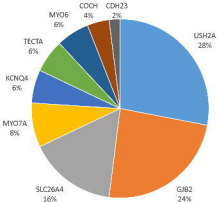| Pathogenic Criteria	
Rule	**Rule Description**
PVS1	Null variant in a gene with established LOF as a disease mechanism; see PVS1_Strong, PVS1_Moderate, PVS1_Supporting for reduced evidence applications
PVS1_Strong	See PVS1 flow chart for PVS1_Strong variants in gene where LOF is a known mechanism of 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.

Table 2. Most common pathogenic variants in hearing loss.

Variant	LMM (Allele Frequency; Allele Ratio)	MORL (Allele Frequency)
GJB2, c.35delG	7.2% (198/2750)	4.77%
GJB2, c.109G>A p.(Val37Ile)	4.4% (120/2750)	2.01%
GJB2, c.101T>C p.(Met34Thr)	2.1% (57/2750)	1.91%
USH2A, c.2299delG	1.2% (46/3774)	0.65%

LMM: Partners Laboratory for Molecular Medicine; MORL: Iowa Molecular Otolaryngology and Renal Research Laboratories. The higher frequency for each of the above grey shaded variants was used to specify the population frequency rules. RefSeq transcripts are NM_004004.5 for *GJB2* and NM_206933.2 for *USH2A*.

Table 3. Allele frequency cutoffs for dominant and recessive hearing loss disorders.

	ACMG-AMP Criteria	MAF	Preval.	Allelic Het.[†]	Penetrance
AUTOSOMAL RECESSIVE	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%
	BS1	≥0.0002 (0.02%)	1/150	5%	80%
	PM2	≤0.00002 (0.002%)	Can apply PM2_Moderate if MAF is an order of magnitude below BS1 (ie ≤0.002%);		

Abbreviations: Preval. = Prevalence. Allelic Het. - Allelic heterogeneity. [†]Allelic heterogeneity encompassed both genetic and allelic heterogeneity because allele frequencies were derived from hearing loss cases spanning all genetic etiologies.

Table 4a: Recommendations for PP1 (segregation evidence)

	General Recommendations		
	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 4b	See Table 4b	See Table4b

Table 4b: Recommendations for autosomal recessive segregation evidence (PP1)

		General Recommendations (Phenocopy not an issue)										
		Unaffected Recessive Segregations										
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
	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
Affected segregations	**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

Affected segregations are counted in rows and unaffected segregations in columns. Affected segregations are affected family members in whom biallelic compound heterozygous or homozygous variants segregates. 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.

Each cell shows the LOD score of each combination of affected and unaffected segregations. LOD scores were calculated using a simplified LOD score formula, as described in Strande et al., 2017.

Table 5a: 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 5b: Recommendation for determining the appropriate ACMG/AMP evidence strength level for de novo occurrence(s)

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

Table 6a: 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 6b: Recommendation for determining the appropriate ACMG/AMP evidence strength level for in trans occurrence(s)

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

Table 7: Recommendations for specification of PP4

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%	28.5% (2/7 cases, LMM unpublished data)

				(Sloan-Heggen et al., 2016)	
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-thrombocytopeniasyn	Unknown

			drome, n.d.)	

PAX3, *SOX10,* *MITF,* *EDN3,* *EDNRB*	Waardenburg syndrome	**Two or more of the following:**
		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. (X-linked mixed deafness with	≥26.7% Testing must include deletion/duplication analysis including 5' promotor region.

			perilymphatic gusher, n.d.).	
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 (Chudley-McCullough syndrome, n.d.)	>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 female*s.* (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.

Table 8: Variant pilot genes and applicable gene-specific ACMG/AMP criteria

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		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		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