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Unique Aspects of Sequence Variant Interpretation for Inborn Errors of Metabolism (IEM): The ClinGen IEM Working Group and the Phenylalanine Hydroxylase Gene

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Abstract

The ClinGen Inborn Errors of Metabolism Working Group was tasked with creating a comprehensive, standardized knowledge base of genes and variants for metabolic diseases. Phenylalanine hydroxylase (PAH) deficiency was chosen to pilot development of the Working Group's standards and guidelines. A *PAH* variant curation expert panel (VCEP) was created to facilitate this process. Following ACMG-AMP variant interpretation guidelines, we present the development of these standards in the context of *PAH* variant curation and interpretation. Existing ACMG-AMP rules were adjusted based on disease (6) or strength (5) or both (2). Disease adjustments include allele frequency thresholds, functional assay thresholds, and phenotype-specific guidelines. Our validation of *PAH*-specific variant interpretation guidelines are presented using 85 variants. The *PAH*VCEP interpretations were concordant with existing interpretations in ClinVar for 69 variants (81%). Development of biocurator tools and standards are also described. Using the *PAH*-specific ACMG-AMP guidelines 714 *PAH* variants have been curated and will be

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submitted to ClinVar. We also discuss strategies and challenges in applying ACMG-AMP guidelines to autosomal recessive metabolic disease, and the curation of variants in these genes.

Keywords

ClinGen; PAH deficiency; Inborn errors of metabolism; variant interpretation; phenylalanine hydroxylase

Introduction

The Clinical Genome Resource (ClinGen) is an international collaboration funded by the National Institutes of Health to advance understanding of genomic variation, thus facilitating improvements in genomic diagnosis. ClinGen launched in 2013 with the goal of implementing evidence-based consensus for curating genes and DNA sequence variants. Several working groups were established within ClinGen, including clinical domain working groups (CDWG). One specific goal of ClinGen is to cultivate teams of experts in various clinical domains to evaluate the clinical validity of gene-disease relationships and the pathogenicity of DNA sequence variants. These CDWG are implementing standardized protocols for gene and variant annotation related to the specific disease domain. Thus, the ClinGen Inborn Errors of Metabolism (IEM) Working Group formed in 2013 to curate IEM genes and variants.

The goals of ClinGen relevant for the IEM CDWG include sharing genomic and phenotypic data for clinical and research use, standardization of annotation and interpretation of genomic variants, use of evidence-based expert consensus for gene and variant curation, and assessing the medical actionability of genes and variants (Rehm, 2015). Following these goals, the IEM CDWG chose Phenylalanine hydroxylase (PAH) deficiency as a pilot for standardization of variant interpretation and curation. PAH deficiency has a prominent historical role as one of the first IEM to be recognized and understood, is universally included in newborn screening, and has well-recognized diagnostic criteria. *PAH* is the only gene known to cause PAH deficiency, although defects in tetrahydrobiopterin (BH₄) metabolism (discussed below) and the co-chaperone *DNAJC12* are known to cause hyperphenylalaninemia (HPA) as well.

PAH deficiency is an autosomal recessive IEM caused by biallelic mutations in *PAH* leading to deficient PAH enzyme. PAH deficiency, commonly referred to as PKU in the classic form, is one of the more common serious genetic conditions, with an incidence of 1 in 13,500 to 19,000 births in the United States (NIH Consensus Development Panel, 2001; Scriver, 2001). PAH converts the amino acid phenylalanine to tyrosine in the liver. In PAH deficiency, phenylalanine accumulates in blood and the central nervous system, leading to severe or profound intellectual disability if untreated. However, Bickel's discovery (Bickel, 1953) that a phenylalanine-restricted diet prevented intellectual disability, and Guthrie's development of newborn screening for PKU (Guthrie, 1962) have led to the successful treatment of thousands of individuals. PAH deficiency is typically diagnosed by measurement of elevated blood phenylalanine levels in the absence of other causes (disorders of BH₄ metabolism, *DNAJC12* deficiency, or liver disease). PAH deficiency is

often classified (from most to least severe) as classic PKU, moderate PKU, mild PKU, or HPA depending on the maximum blood phenylalanine levels in untreated individuals (Camp, 2014). BH₄ defects collectively account for approximately 2% of individuals with HPA in most populations implying that the vast majority of individuals with persistent HPA have PKU (Regier, 2017). Disorders of BH₄ metabolism causing HPA are genetic disorders caused by deficiencies of enzymes involved in BH₄ synthesis or regeneration. These defects differ from PAH deficiency in that treatment with a phenylalanine-restricted diet is not sufficient to prevent intellectual disability and central nervous system dysfunction. The genes encoding enzymes involved in BH₄ metabolism are distinct from *PAH*, and this must be taken into account during development of curation guidelines for *PAH*.

Two pathogenic or likely pathogenic variants in *PAH* are found in 97–99% of affected patients (Regier, 2017 and personal communication, McKenna Kyriss, PhD, PreventionGenetics). However, the allelic heterogeneity in *PAH* makes it difficult to definitively classify variant pathogenicity. Over 1000 variants have been described in *PAH*, corresponding with 2,589 different genotypes (www.BIOPKU.org, the Phenylalanine Hydroxylase Knowledgebase). More than half of the genotypes (53%) are reported in a single patient. In addition to the use of genetic testing for diagnostic confirmation, testing is also useful in classifying the severity of PAH deficiency (Okano, 1991), predicting therapeutic response, excluding BH₄ disorders as the cause of HPA, and for genetic counseling. Here, we describe the process undertaken to create an infrastructure for the development of rules to assess the pathogenicity of *PAH* variants and the application of the rules for *PAH* variant curation as a model for other inborn errors of metabolism.

Methods

ClinGen IEM CDWG and the *PAH* Variant Curation Expert Panel

The IEM Executive Committee was constituted with 18 professionals from a broad range of specialties in order to represent the clinical domain across key expertise categories (clinical, research, molecular laboratory, etc.), including clinical geneticists, clinical biochemical geneticists, funding agency partners, genetic counselors, laboratory geneticists, and human genetics researchers. A small leadership group is composed of three co-chairs, a ClinGen coordinator, and a Principal Investigator liaison from one of the three NHGRI cooperative grants. The IEM Executive Committee meets regularly via biweekly teleconferences, and semi-annually at professional meetings for progress updates, communication across the consortium, and operational decision-making.

The IEM CDWG created a variant curation expert panel (VCEP) to direct and oversee variant curation for *PAH*. The *PAH*VCEP is an international team with similar makeup as the IEM CDWG. This Expert Panel formed from a subset of IEM Executive Committee members and recruited additional expert reviewers and curators. Approval of VCEPs is overseen by ClinGen and consists of four steps: (1) Defining the group/members; (2) Develop classification rules; (3) Pilot rules; and (4) Approval of the Expert panel by ClinGen (<https://www.clinicalgenome.org/expert-groups/>). The *PAH*VCEP developed *PAH* specifications of ACMG-AMP criteria, and set a process for variant review and classification

considering biochemical and molecular aspects of the disorder. Final approval of the *PAH* VCEP was received from the ClinGen Steering Committee.

Adaptation and Specification of ACMG-AMP Variant Interpretation Criteria for *PAH*

Following ACMG-AMP standards and guidelines for the interpretation of sequence variants (Richards, 2015), each criterion was reviewed by the *PAH* VCEP and made specific for *PAH* deficiency. Criteria justifications include *PAH*-specific adjustments of existing ACMG-AMP criteria for additional evidence, and criteria not applicable to *PAH*. Any remaining criteria not specific to *PAH* were used as recommended. Additionally, recommendations for using ACMG-AMP criteria from the ClinGen Sequence Variant Interpretation (SVI) working group were incorporated (Biesecker, 2018; <https://www.clinicalgenome.org/working-groups/sequence-variant-interpretation/>). The final *PAH* specific guidelines were approved by the SVI workgroup.

Assessment of Existing *PAH* variants in ClinVar

The current state of variant annotation of *PAH* in ClinVar as of March 2018 was compiled from ClinVar Miner, a tool that enables detailed exploration of ClinVar submissions and provides tools for resolution of conflicting interpretation (Henrie, 2018).

Selection of *PAH* Variants for Validation of Interpretation Guidelines

Eighty-five pilot variants were selected to validate the *PAH*-specific variant interpretation guidelines (Supp. Table S1). We included *PAH* variants with multiple submissions in ClinVar (7 B/LB, one VUS, 53 P/LP, three conflicting interpretations), variants to assess our allele frequency cutoffs, and additional VUS and B/LB variants. These variants cover various types of alterations: missense, nonsense, splicing, synonymous, frameshift, inframe indel, initiation codon, and intronic (Figure 1).

Variant Review and Classification

PAH variant curation began using 895 *PAH* missense variants listed in the professional version of Human Gene Mutation Database (HGMD). In the pilot phase of curation and variant review, *PAH* VCEP member-duos curated 20 *PAH* variants using the *PAH*-specific guidelines (RM/YS, WC/AF, RM/MP, YS/MP). Evidence used for each variant and the interpretation was presented to the *PAH* VCEP for review and final classification. This process continued for 185 variants, with curation conducted by a single experienced curator (YS, HB, DZ, AT, WS, AL, JL, RM), and variant review and classification approval by the *PAH* VCEP (on workgroup calls). New curators were trained and paired with an experienced curator.

A second phase of curation incorporated variants and data from BioPKU, for a total of 961 variants as of September, 2016. Nonsense variants and large deletion variants were batch curated. Due to the large volume of *PAH* variants, variant presentation to the *PAH* VCEP was deemed necessary when the curator wanted input from the group, or for variants with interpretations that conflict with the existing interpretation in ClinVar. With the availability of the Variant Curation Interface, final approval of variants will require review by two *PAH*

VCEP members before submission to ClinVar (e.g., provisional and approved interpretations).

Development of Biocurator Tools and Standards

A curation Standard Operating Procedure (SOP) was created for standardization and training. Our variant assessment framework was adapted from Duzkale, 2013. This describes five main steps of curation: (1) Transcription of *PAH* databases (BioPKU and PAHdb); (2) Assessment of allele frequency; (3) Predicted effect on protein structure or splicing; (4) Clinical significance from HGMD and ClinVar (for curator reference); and (5) Search of primary literature. Publicly available data sources are listed in Supp. Table S2.

A password-protected online open-source relational database management system was created in 2015 and stores *PAH* variant curation data. Specifically, a MySQL database, managed and accessed by PHPMyAdmin was created as an online curation workspace that allows for live editing and data input. This enabled self-assignment of variants for curation to avoid duplicated efforts while optimizing curator workload, and allowed for detailed tracking of the curation effort. Data can be exported and archived.

ClinGen Variant Curation Interface

The ClinGen Variant Curation Interface (VCI) is a publically available interface (<https://curation.clinicalgenome.org/>) developed at the Stanford University School of Medicine that streamlines interpretation of variants through a web-based portal, utilizing the 2015 ACMG/AMP Sequence Variant Interpretation Guidelines. The VCI came online in October 2016, and the *PAH*VCEP incorporated it into our existing workflow. Users begin a variant interpretation using a ClinVar Variation ID or a ClinGen Allele Registry (<http://reg.genome.network/>) ID (CAid). The VCI streamlines variant curation by aggregating and displaying information from external resources such as dbSNP IDs, population data, *in silico* predictors, and genome and transcript information. Evidence specific to ACMG/AMP criteria may also be manually curated and saved into the VCI by the user; this evidence then becomes visible to all users. The VCI allows evaluation of evidence criteria and calculates pathogenicity in real-time according to the ACMG/AMP guidelines. A future goal of the VCI is to facilitate variant submission to ClinVar after approval by the Expert Panel. While the VCI aids in collecting variant level evidence and calculation of variant pathogenicity, the *PAH*VCEP database provides a way to store all *PAH* variants for tracking of completion and assignment to curators.

Results

Existing ACMG-AMP sequence variant interpretation rules were adjusted for *PAH* based on disease (6) or strength (5) or both (2) (Table 1). Disease adjustments include allele frequency thresholds, functional assay thresholds, and phenotype-specific guidelines.

ACMG-AMP Criteria Specification for *PAH*

PS2: *de novo* occurrence (paternity confirmed)—In *PAH* deficiency, as with other autosomal recessive disorders, *de novo* variants are extremely rare. However, when it occurs,

PS2 is only applicable when the case also has another known pathogenic variant in *trans* with the *de novo* variant.

PS3: *in vitro* functional studies—The *PAHV*CEP reviewed all described functional assays and recommended a well-established *in vitro* PAH enzyme activity assay used by multiple investigators (Okano, 1991; Gjetting, 2001; Kim, 2006; Pey, 2003; Trunzo, 2016; Waters, 1998) that expresses the variant allele in cultured cells, and measures the enzyme activity in comparison to the wild type enzyme activity. Residual mutant enzyme activity of 0–50% can be used as PS3 evidence. If there are no reports of a variant having a deleterious impact using other assays (e.g., reaction kinetics, protein aggregation and folding), use of BS3_supporting could be applied for variants with mutant *in vitro* enzyme activities similar to wild type.

PM3: Detected in *trans* with a pathogenic variant—This moderate pathogenic criterion has been increased in strength when the variant of interest has been seen multiple times in *trans* with separate known pathogenic variants in probands with PAH deficiency, as recommended by the SVI working group. It is upgraded to strong (PM3_Strong) when observed in *trans* in two compound heterozygotes with two known pathogenic or likely pathogenic variants. It can be used as very strong evidence (PM3_Very Strong) when a *PAH* variant is found in compound heterozygosity four times with three different known pathogenic or likely pathogenic variants. This evidence may also be downgraded (PM3_supporting) in order to include compound heterozygosity with two separate VUS, or when observed twice in the homozygous state in affected individuals.

Allele frequency (BA1, BS1, PM2)

In PAH deficiency, allele frequency was set for BA1 at 1.5% or 0.015 using an allele frequency calculator (<https://www.cardiodb.org/allelefrequencyapp/>). This calculator uses disease prevalence, genetic heterogeneity of 90% (to account for defects of BH₄ metabolism), and penetrance of 80% (to account for individuals who come to attention after becoming clinically symptomatic). The allele frequencies of the most common *PAH* pathogenic variants from ExAC and gnomAD were used to assess the BS1 (Allele frequency greater than expected for disease) threshold. The most frequent *PAH* pathogenic variant, c. 1222C>T (p.Arg408Trp), has a minor allele frequency of 0.1728% in gnomAD, so 0.002 or 0.2% was used for BS1. The PM2 threshold was then set at an order of magnitude lower, allele frequency of 0.0002 or 0.02%, as recommended by the SVI working group. We then assessed other *PAH* variants with frequencies in these ranges (Figure 2). Although six additional known pathogenic variants have allele frequencies between 0.0002–0.002, they remained pathogenic even if PM2 was not applied (Table S1). Similarly, known benign *PAH* variants all had allele frequencies over 0.002, and were closer to 0.015 and above.

PP4: Patient Phenotype—The supporting pathogenic evidence, PP4, was applied to *PAH* to reflect the clinical diagnostic criteria for PAH deficiency: phenylalanine concentration persistently above 120 μmol/L (2 mg/dL) (Vockley, 2014). If either normal urine pterins and normal DHPR activity or DNA sequencing of genes in the BH₄ cofactor metabolism pathway is performed, this evidence is upgraded to PP4_Moderate.

Existing *PAH* Variants in ClinVar

There are a total of 655 *PAH* variants in ClinVar (Figure 3), of which 536 were submitted by the DeBelle Laboratory for Biochemical Genetics and corresponds with the variants from PAHdb (clinical significance was not provided). Clinical significance is available for 322 *PAH* variants. Of these, 91 *PAH* variants have multiple submitters (8 benign/likely benign, one uncertain significance (US), 82 pathogenic/likely pathogenic). In total, there are seven *PAH* variants with clinically significant conflicting interpretations (benign vs. pathogenic vs. US).

Fourteen groups have submitted ClinVar annotations for *PAH* with their method as clinical testing, which can be used as a proxy for submissions from diagnostic laboratories. There are three conditions currently annotated to *PAH* by these 14 submitters, with 154 variants submitted for Phenylketonuria, 84 for “*not provided*” and 37 for “*not specified*”. *Not provided* is used when the submitter does not know the clinical phenotype and *not specified* is used to describe the case where a variant is annotated as benign for all highly penetrant Mendelian diseases.

While there are 46 variants showing some degree of conflicting interpretation when evaluated across all submitters, this number falls to 19 when selecting only for clinical testing as the method. In the ClinVar submissions from clinical testing, there are 133 variants with a single submission, and 58 variants with at least two submissions and no conflicting interpretations. There are 114 assertions of pathogenic (P), 51 likely pathogenic (LP), 37 uncertain significance (US), 13 likely benign (LB) and 14 benign (B), totalling 229 assertions for a total of 210 variants. Further exploration of the variants with conflicting interpretation reveals that there are three variants with a clinically significant conflict. These variants are annotated as both US and/or P/LP, and are displayed in Table 2, along with the interpretation of the *PAH*VCEP. There are 16 variants that were interpreted within confidence limits (e.g., benign vs. likely benign), 12 are P/LP and four are B/LB.

Validation of the *PAH*-specific ACMG-AMP variant interpretation guidelines

Using the *PAH*-specific variant interpretation guidelines, 67 validation variants were classified by the *PAH*VCEP as pathogenic (50) or likely pathogenic (17), 9 were VUS, 9 were likely benign (3) or benign (6) (Figure 4). The commonly used criteria, as shown in Figure 5, are PM2 (53/85), PP4_Moderate (44/85), PP3 (43/85), and PM3 (36/85). The criteria for benign evidence were used less frequently, reflecting the pathogenic spectrum of the variants selected for validation. The most commonly used criteria, pertinent to population frequencies, were PM2, BA1 and BS1. Since plasma phenylalanine concentrations were often reported for patients with *PAH* deficiency, PP4 and PP4_Moderate were used for the majority of the variants (60/85). In addition, forty-five variants have data from *in vitro* functional studies, allowing the usage of PS3 or BS3_Supporting.

Among the 85 *PAH* variants used for validation, eighty-four have interpretations in ClinVar. Among these, 69 are concordantly classified with the *PAH*VCEP. All variants with multiple submitters (n=61) had *PAH*VCEP interpretations that were clinically concordant with their interpretations in ClinVar (Supp. Table S1). Eleven of the variants have clinically discordant

interpretations between *PAH*VCEP and ClinVar submitters (Table 3). For the three variants with conflicting interpretations (P/LP vs VUS) in ClinVar, we classified them as likely pathogenic using the *PAH*-specific guidelines (Table 2).

***PAH* Variant Curation by the *PAH* VCEP**

Curation according to the *PAH*-Specific Guidelines has been completed on 714 *PAH* variants, with 15 in progress as of April, 2018. Of the 714 variants, 256 are absent from ClinVar. Of the 458 completed variants present in ClinVar, 236 do not have a clinical assertion, 198 have clinically concordant interpretations (185 LP/P, five VUS, eight B/LB), five are for variants with conflicting interpretations in ClinVar, and 19 are clinically discordant/conflicting (Figure 6).

Discussion

Lumping vs. Splitting

When deciding how to curate variants in the *PAH* gene, the question arose whether to associate the variant with a specific phenotype (e.g., HPA or classic PKU), which can be referred to as “splitting” the gene-disease association. There is an abundance of literature that has attempted to categorize the *PAH* genotype-phenotype correlation, while acknowledging that two alleles contribute to the phenotype (Goldberg, 1998; Guttler, 1994; Kayaalp, 1997; Okano, 1991). Splitting gene-disease phenotypes may be more applicable for genes with distinct syndromes and differing molecular mechanisms ([www.https://www.clinicalgenome.org/working-groups/lumping-and-splitting/](https://www.clinicalgenome.org/working-groups/lumping-and-splitting/)). The *PAH*VCEP decided to “lump” together the described *PAH* deficiency phenotypes (classic PKU, moderate PKU, mild PKU, and HPA), and not to associate *PAH* variants with a specific phenotypic severity. This follows the ACMGG guidelines for *PAH* deficiency, which recommend describing all affected individuals with a plasma phenylalanine concentration above 120 μ mol/L (2 mg/dL) as having *PAH* deficiency (Vockley, 2014). In part, this reflects the need to provide an interpretation for clinically benign forms of HPA that are still detected by newborn screening. Variant annotation can help define how an initially mild phenotype (HPA) may progress. Some patients with subclinical serum Phe levels in the HPA range remain static, in contrast to others whose initial subclinical Phe levels increase with age. These two groups may possibly be separated by genotype. Thus, variant analysis is of importance for all classes of *PAH* deficiency. In addition, about 75% of patients with *PAH* deficiency are compound heterozygous; given the number of pathogenic variants in the *PAH* gene and the possible genotype combinations there is a continuum of phenotypes. Pathogenic variants are associated with a range of phenotype severity depending on the second allele, and a definitive phenotype can only be assigned to a variant observed in the homozygous state. Thus ‘splitting’ by phenotype severity would not adequately represent the genotypic spectrum. BioPKU includes a genotype-phenotype database (BioPKUdb) which is helpful for clinicians to predict patient phenotypes correlating to known genotypes. This approach of lumping the gene-disease phenotype will apply to a majority of IEM, where phenotypes differ due to varying severity of enzyme inpairment.

Using a Biochemical Phenotype

A challenge of applying existing ACMG-AMP criteria was to adequately represent current clinical diagnostic methods for PAH deficiency using the variant interpretation evidence categories. Many IEM are detected by newborn screening before symptoms, or a typical phenotype, develop. Instead of using a clinical phenotype of untreated PAH deficiency (e.g., intellectual disability) as evidence for PP4 criteria, we chose to use a biochemical phenotype of elevated plasma phenylalanine levels to be consistent with current ACMGG diagnostic criteria for PAH deficiency (Vockley, 2014). The existing ACMG-AMP PP4 criterion was increased in strength (PP4_Moderate) with assessment of BH₄ cofactor metabolism in a patient, to reflect a more specific phenotype consistent with PAH deficiency. In certain populations (e.g., Saudi Arabia and Taiwan), BH₄ cofactor metabolism may account for the majority of cases of elevated phenylalanine. There was a discussion of applying the ACMG-AMP PS3 criteria to plasma phenylalanine levels. However, this measurement is not directly assessing the *PAH* gene product. Assessment of phenylalanine hydroxylase activity requires a liver biopsy, which is not performed clinically. Enzyme analysis is not used clinically in the diagnosis of PAH deficiency because accurate, less invasive methods of diagnosis are available. For other IEMs where direct analysis of the enzyme is not available, using the PP4 criteria with possible strength modifications allows inclusion of important phenotypic evidence.

Applying Functional Assays

An established assessment of *PAH* variant pathogenicity are *in vitro* enzyme assays (Okano, 1991; Gjetting, 2001; Kim, 2006; Pey, 2003; Trunzo, 2016; Waters, 1998). They directly reflect the specific variant, and are not subject to confounding effects of the other allele. These satisfy the functional assay criteria (PS3), but certain aspects of these assays were evaluated. One concern was that these assays are research studies and are not performed in a regulated environment (e.g., CLIA lab test or using a commercially available kit). So consideration was given to mutant enzyme activity studies in triplicate. Typically, these experiments are performed with transiently transfected mammalian host cells (e.g., COS cells). More recent studies have better standardized mutant PAH enzyme activity assays using mammalian COS cells and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS-MS) for quantification of phenylalanine and tyrosine (Heintz, 2012; Shen, 2016; Trunzo, 2016). Also, a cut-off had to be determined for standardization as to what constituted a deleterious effect on the enzyme. Gjetting et al. data showed *in vitro* mutant enzyme activity ranging between 0 and 74% for variants typically associated with classical PKU *in vivo*, and Waters et al. reported *in vitro* residual activities of 0–75% for their mutants. However, variants associated with milder clinical disease can have *in vitro* activity closer to wild type (9–128%, Gjetting, 2001). Pey et al. grouped their mutants based on *in vitro* activity of 0%, 10–70%, and 80–100%. A conservative cut-off of 0–50% residual mutant enzyme activity *in vitro* was decided for use as PS3 evidence. Caution should be used in applying mutant *in vitro* enzyme activities similar to wild type for BS3 criteria, as these variants may have alternative mechanisms of disease that are better assessed with other less common assays (Pey, 2003). If there are no reports of a variant having a deleterious impact using other assays (e.g., reaction kinetics, protein aggregation and folding), use of BS3_supporting could be applied for these variants. Our use of this

category of functional evidence for non-clinical testing provides an example for other IEM where direct assay of the enzyme is difficult or uncommon.

Allele Frequency Thresholds

Typically the *PAH* variants reported in affected individuals are absent from the population databases, or at an extremely low frequency. However, a cutoff for allele frequency was necessary in order to standardize curation. ACMG-AMP guidelines state that allele frequency for pathogenic variants in recessive disease should be consistent with disease incidence/carrier frequency (Richards, 2015), suggesting an allele frequency for a *PAH* variant of 2.0% meets criteria for BS1. However, this was adjusted to 0.2–1.5% using an allele frequency calculator and frequent *PAH* variants with known pathogenicity. This lower threshold reflects the genetic heterogeneity of *PAH* variants, and the lack of a frequent pathogenic variant that approaches the carrier frequency. When approaching the modification of ACMG-AMP criteria for recessive disease, allele frequency thresholds could be assessed using known pathogenic and benign variants, and a calculator that takes into account disease specific aspects.

Expanding the Number of *PAH* Variants with Clinical Interpretation

The efforts of the ClinGen IEM CDWG and *PAH* VCEP illustrated here demonstrate our progress in achieving our goals of evaluating the pathogenicity of known *PAH* variants according to our *PAH*-specific ACMG-AMP guidelines and adding to the breadth of curated *PAH* variants. Although *PAH* variants from one of the major *PAH* databases (PAHdb) have been submitted to ClinVar, the clinical significance was not incorporated into that submission. Additionally, *PAH* variants in the BioPKU database have not yet been submitted to ClinVar. We have partnered with BioPKU to curate the *PAH* variants not currently in ClinVar (n=256 as of March, 2018) and facilitate their submission to ClinVar. The *PAH* VCEP will add clinical interpretation to almost half (492/1044) of all reported *PAH* variants after variant curation and ClinVar submission is completed (total number of variants from BioPKU as of March 28, 2018).

Limitations

One of the challenges was to modify the ACMG-AMP criteria for variant interpretation for *PAH*, for which a well-defined biochemical phenotype exists. The pathogenicity of a variant can be assigned based on the effect on the protein. Hence, the effect on protein activity is estimated from the concentration of phenylalanine in plasma/serum/blood spots. This depends not only on enzymatic activity but also on phenylalanine intake which cannot always be measured accurately due to variable dietary compliance and reporting. In addition, for several *PAH* variants the lack of evidence in the literature that other causes of elevated phenylalanine had been excluded limits our ability to properly assess their pathogenic criteria. Finally, we are just now entering the era of genome sequencing and the presence of additional variants in the regulatory regions of the gene in *cis* with the observed exonic variant cannot be excluded.

Conclusion and Future Directions

The ClinGen IEM CDWG was tasked with creating a comprehensive, standardized knowledge base of genes and variants for metabolic diseases. PAH deficiency was chosen to pilot development of the Working Group's standards and guidelines. The specifications of the guidelines outlined above and application of these guidelines to classify over 1,000 variants at the *PAH* locus has led to a near complete characterization of pathogenicity for what is one of the most mutated genes in humans. This exemplar creates a pathway forward in curating the hundreds of distinct inborn errors of metabolism, providing guidance in weighing the impact on pathogenicity scoring of disease-specific analytes, and incorporating additional biochemical phenotypes and *in vitro* enzyme assays. While each IEM has its own unique features, our experience will help inform future efforts with these other autosomal recessive conditions. With completion of the *PAH* pilot the IEM CDWG has created sub-groups that are tackling pathway-specific domains (e.g., fatty acid oxidation disorders, aminoacidopathies, and lysosomal storage disorders), assembling teams with domain-specific clinical expertise and biocuration capacity, followed by completing the required gene curation before addressing variant curation. A broad expansion of these sub-groups will be required to complete these tasks in an achievable time frame.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Types of alterations for 85 *PAH* variants selected for validation. *PAH* variants selected to validate the *PAH*-specific variant interpretation guidelines include a range of genetic alterations.

(a) Known pathogenic *PAH* variants **(b) Known benign *PAH* variants** **Figure 2.**

Setting allele frequency thresholds using known *PAH* variants. **A:** Known pathogenic *PAH* variants. BS1 threshold (allele frequency greater than expected for the disorder) was set at an allele frequency of 0.002 based on the most common *PAH* pathogenic variant p.Arg408Trp. PM2 threshold (extremely low frequency in a general population cohort) was set at an order of magnitude lower (0.0002). **B:** Known benign *PAH* variants. BA1 threshold was set at an allele frequency of 0.015 using an allele frequency calculator. Five known benign variants have an allele frequency greater than 0.015. The p.Tyr414= variant has an allele frequency of 0.01361 in the European (Non-Finnish) population. RefSeq NM_000277.2 or GenBank U49897.1. ENF=European Non-Finnish population. Pop Max=Population with the maximum allele frequency.

Figure 3.

Existing *PAH* variants in ClinVar. There are a 655 *PAH* variants in ClinVar, of which 536 were submitted by the DeBelle Laboratory for Biochemical Genetics and corresponds with the variants from PAHdb (clinical significance was not provided). Clinical significance is available for 322 *PAH* variants, with 210 coming from clinical testing submissions and 246 from literature and/or research submitters. Of these, only 91 *PAH* variants have multiple submitters. While there are 46 variants showing conflicting interpretations, the majority of those are based on literature and/or research submissions. Only 3 *PAH* variants with submissions from clinical testing have clinically significant conflicting interpretations. Totals in the left column correspond to variants submitted from literature and research. Totals in the right column correspond to variants submitted from clinical testing. [†]Number of variants in both columns are not additive due to variants with multiple clinical, literature, and/or research submissions. P=Pathogenic, LP=Likely pathogenic, US= Uncertain significance, B=Benign, LB=Likely benign.

Figure 4. *PAHVCEP* interpretation summary for the 85 *PAH* validation variants. Using the *PAH*-specific variant interpretation guidelines, 67 variants were classified by the *PAHVCEP* as pathogenic (50) or likely pathogenic (17), 9 were variants of uncertain significance (VUS), 9 were likely benign (3) or benign (6).

Figure 5. Summary of the *PAH*-specific ACMG-AMP variant interpretation criteria applied to the 85 *PAH* validation variants. The commonly used criteria include *PAH*-specific rules: PM2 (53/85), PP4_Moderate (44/85), and PS3 (43/85).

Figure 6.

Comparison of *PAH*VCEP completed variant interpretations with existing interpretations in ClinVar (n=217). Curation according to the *PAH*-Specific Guidelines is complete for 217 *PAH* variants that have an existing interpretation in ClinVar. 198 variants have clinically concordant interpretations (185 LP/P, 5 VUS, 8 B/LB), 5 are for variants with conflicting interpretations in ClinVar, and 19 are clinically discordant/conflicting. P=Pathogenic, LP=Likely pathogenic, VUS= Uncertain significance, B=Benign, LB=Likely benign.

Table 1.

ACMG-AMP Criteria Specific for Phenylalanine Hydroxylase (*PAH*) Variants

PATHOGENIC CRITERIA		
Criteria	Criteria Description	Specification
VERY STRONG CRITERIA		
PVS1	Null variant (nonsense, frameshift, canonical +/-1 or 2 splice sites, initiation codon, single or multi-exon deletion)	None
PM3_Very Strong	Detected in <i>trans</i> with a pathogenic variant •4 compound heterozygotes with 3 P/LP variants OR •2 compound heterozygotes with 2 P/LP variants AND 4 homozygotes OR •3 compound heterozygotes with 2 P/LP variants AND 2 homozygotes	Strength
STRONG CRITERIA		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change	None
PS2	<i>De novo</i> (paternity confirmed) in a patient with disease and no family history	None
PS3	Well-established <i>in vitro</i> functional studies supportive of a damaging effect •Mutant PAH enzyme activity assay demonstrating enzyme activity <50% •RT-PCR evidence of missplicing for non-canonical intronic variants	Disease-specific
PM3_Strong	Detected in <i>trans</i> with a pathogenic variant •2 Compound heterozygotes with 2 P/LP variants OR •1 Compound heterozygote with P/LP variant AND 2 homozygotes	Strength
PP1_Strong	Co-segregation with disease in multiple affected family members: •3 affected segregations + 0 unaffected segregations OR •2 affected segregations + 3 unaffected segregations	Strength
MODERATE CRITERIA		
PM2	Absent/rare in controls from an ethnically matched cohort population sample Allele frequency threshold: <0.0002 (0.02%)	Disease-Specific
PM3	Detected in <i>trans</i> with a pathogenic variant	None
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants	None
PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before	None
PM6	Assumed <i>de novo</i> , without confirmation of paternity and maternity	N/A
PP1_Moderate	Co-segregation with disease in multiple affected family members •2 affected segregations + 0 unaffected segregations	Strength
PP4_Moderate	Plasma phenylalanine > 120 $\mu\text{mol/L}$ (2 mg/dL), with normal BH ₄ cofactor metabolism (normal urine pterins and DHPR activity, or sequencing of genes in the BH ₄ cofactor metabolism pathway)	Strength; Disease-Specific
SUPPORTING CRITERIA		
PP1	Segregation with disease in 1 affected family member + 3 unaffected segregations	Disease-Specific
PP2	Missense variant in a gene that has a low rate of benign missense variation and in which missense variants are a common mechanism of disease	N/A
PP3	Multiple lines of computational evidence support a deleterious effect	None
PP4	Persistently abnormal plasma Phe without excluding BH ₄ deficiencies	Disease-Specific
PM3_Supporting	Detected in <i>trans</i> with another variant: •2 compound heterozygotes with VUS in <i>trans</i> •2 homozygotes (allele drop out ruled out)	Strength
BENIGN CRITERIA		
Criteria	Criteria Description	Specification

STAND ALONE CRITERIA		
BA1	Allele frequency above 0.015 (1.5%)	Disease-Specific
STRONG CRITERIA		
BS1	Allele frequency greater than expected for disease (0.002–0.015 or 0.2–1.5%)	Disease-Specific
BS2	Observed in the homozygous state in a healthy adult	None
BS4	Lack of segregation in affected members of a family	None
SUPPORTING CRITERIA		
BP1	Missense variant in gene for which primarily truncating variants are known to cause disease	N/A
BP3	In-frame deletions/insertions in a repetitive region without a known function	N/A
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product	None
BP5	Variant found in a case with an alternate molecular basis for disease	None
BP6	Reputable source recently reports variant as benign, but the evidence is not available to perform an independent evaluation	N/A
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved	None
BS3_Supporting	Well-established <i>in vitro</i> functional studies show no damaging effect on protein function Enzyme activity >85%	Strength; Disease-Specific

Criteria for classifying pathogenic and benign *PAH* variants. Disease-Specific: Disease-specific modifications based on what is known about *PAH*; Strength: Increasing or decreasing strength of criteria based on the amount of evidence; None: no changes made to existing criteria definitions; N/A: not applicable for *PAH*.

Table 2.*PAH* Variants with Clinically Significant Conflicting Interpretations in ClinVar

<i>PAH</i> Variant	Protein Effect	ClinVar ID	PM2	PM3	PP4	Interpretation in ClinVar	<i>PAH</i> VCEP Interpretation
c.500A>T	p.Asn167Ile	92743	X	PM3_S	PP4_M	LP(1); US(1)	LP
c.506G>A	p.Arg169His	102706	X	PM3_S	PP4_M	LP(3); US(1)	LP
c.842+3G>C		102871	X	PM3_S	PP4_M	P(2); US(1)	LP

Only 3 *PAH* variants submitted to ClinVar through clinical testing have clinically significant conflicting interpretations. The ClinVar interpretations are displayed along with the *PAH* VCEP interpretation and *PAH*-specific criteria used. Number in parenthesis is number of clinical testing submitters with that interpretation. RefSeq NM_000277.2 or GenBank U49897.1. P=Pathogenic, LP=Likely pathogenic, US= Uncertain significance.

Table 3.Validation *PAH* Variants with Clinically Significant Discordance Between *PAH* VCEP and ClinVar

<i>PAH</i> variant	Protein effect	PM2	PM3	PM4/5 PP3	PP4	Interpretation in ClinVar	<i>PAH</i> VCEP Interpretation
c.205_210dup	p.Pro69_Ser70dup	X		PM4		LP (lit)	US
c.350C>T	p.Thr117Ile	X				LP (lit)	US
c.800A>T	p.Gln267Leu	X		PP3	PP4_M	LP (clin)	US
c.812A>T	p.His271Leu	X		PP3	PP4_M	LP (lit)	US
c.169G>A	p.Glu57Lys	X	X		PP4_M	US (clin)	LP
c.464G>C	p.Arg155Pro	X	PM3	PP3	PP4_M	US (clin)	LP
c.511G>A	p.Gly171Arg	X	PM3_S	PP3	PP4_M	US (clin)	LP
c.520A>G	p.Ile174Val	X	PM3	PP3	PP4	US (clin)	LP
c.535T>A	p.Tyr179Asn	X	PM3	PP3	PP4_M	US (clin)	LP
c.841C>T	p.Pro281Ser	X	PM3_S	PP3	PP4_M	US (clin)	LP
c.1033G>A	p.Ala345Thr	X	PM3	PM5, PP3	PP4_M	US (ref)	LP

11 variants used for validation of the PAH specific guidelines have a *PAH* VCEP interpretation that is clinically discordant with the interpretation in ClinVar. The ClinVar interpretations are displayed along with the PAHEP interpretation and PAH-specific criteria used. (lit): Collection method literature only; (clin): Collection method clinical testing; (ref): Collection method reference population. P=Pathogenic, LP=Likely pathogenic, US=Uncertain significance. RefSeq NM_000277.2 or GenBank U49897.1.