Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework

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ABSTRACT

Background: The American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP) clinical variant interpretation guidelines established criteria (PS3/BS3) for functional assays that specified a "strong" level of evidence. However, they did not provide detailed guidance on how functional evidence should be evaluated, and differences in the application of the PS3/BS3 codes is a contributor to variant interpretation discordance between laboratories. This recommendation seeks to provide a more structured approach to the assessment of functional assays for variant interpretation and guidance on the use of various levels of strength based on assay validation.

Methods: The Clinical Genome Resource (ClinGen) Sequence Variant Interpretation (SVI) Working Group used curated functional evidence from ClinGen Variant Curation Expert Panel-developed rule specifications and expert opinions to refine the PS3/BS3 criteria over multiple in-person and virtual meetings. We estimated odds of pathogenicity for assays using various numbers of variant controls to determine the minimum controls required to reach supporting or moderate level evidence. Feedback from the ClinGen Steering Committee and outside experts were incorporated into the recommendations at multiple stages of development.

Results: The SVI Working Group recommends that evaluators use a four-step process to determine the applicability and strength of evidence of functional assays for use in clinical variant interpretation. These steps are: 1. Define the disease mechanism; 2. Evaluate general classes of assays used in the field; 3. Evaluate validity of specific instances of assays; 4. Apply evidence to individual variant interpretation. We found that a minimum of eight total pathogenic and benign variant controls are required to reach supporting-level evidence and twenty are required to reach moderate-level evidence in the absence of rigorous statistical analysis.

Conclusions: The recommendations and approach to functional evidence evaluation described here should help clarify the clinical variant interpretation process for functional assays. Further, we hope that these recommendations will help develop productive partnerships with basic scientists who have developed functional assays that are useful for interrogating the function of a variety of genes.

KEYWORDS (3-10)

Functional assays; guidelines; variant interpretation

BACKGROUND

The American College of Medical Genetics (ACMG) and the Association for Molecular Pathology (AMP) jointly developed standards and guidelines for assessment of evidence to increase consistency and transparency in clinical variant interpretation [1]. One type of evidence defined in this guideline was the effect of a variant on gene/protein function as determined by a "well-established" functional assay, which provides strong support of a pathogenic or benign impact (rule codes PS3 and BS3, respectively). The full definition is provided in Text Box 1. Functional studies can provide powerful insight into the effect of a variant on protein function and have the capacity to reclassify variants of uncertain significance (VUS) [2], underscoring the need for experimental evidence to be applied accurately and consistently in variant interpretation. However, the ACMG/AMP standards did not provide detailed guidance on how functional evidence should be evaluated, and differences in application of the PS3/BS3 codes represent a major contributor to variant interpretation discordance among clinical laboratories [3].

In response to calls to further standardize variant interpretation [3,4], the Clinical Genome Resource (ClinGen) established the Sequence Variant Interpretation Working Group (SVI) [5] and condition-specific Variant Curation Expert Panels (VCEPs) to refine ACMG/AMP guidelines for each evidence criterion [6]. To date, six VCEPs have published recommendations, including their assay validation requirements and which assays were ultimately approved for PS3/BS3 evidence application [7–12]. The VCEPs vary significantly in how they defined which assays were "well-established" [Kanavy et al., submitted], including consideration of parameters such as experimental design, replication, controls and validation, indicating the subjective nature

of assessing the quality and applicability of functional evidence, potentially leading to discordance in variant classification.

In this manuscript, we detail additional guidance developed by the SVI regarding assessment of the clinical validity of functional studies and the determination of suitable evidence strength levels, with the goal that experimental data cited as evidence in clinical variant interpretation meets a baseline quality level.

METHODS

In November 2018, during the monthly SVI Working Group conference call, we first outlined our goals of defining what constitutes a well-established functional assay and how functional assay evidence should be structured for computation and curation. In this meeting, we presented a preliminary approach to curating functional evidence and important considerations for assay validation. This process was subsequently presented to ClinGen Steering Committee in-person meeting in Seattle, WA, in December 2018 for comments and further refinement. The proposed PS3/BS3 evaluation process was then discussed on the SVI Working Group call in March 2019 and again in-person at the American College of Medical Genetics and Genomics (ACMG) meeting in April 2019. Subsequently, a smaller subgroup developed a final version of these recommendations, which were then approved by the SVI Working Group.

We used curated functional evidence from VCEP-developed rule specifications [Kanavy, et al., submitted] and expert opinions throughout the PS3/BS3 criterion refinement process. Feedback from the broader SVI working group, ClinGen Steering Committee, and outside experts were incorporated into the recommendations at multiple stages of development.

To determine the minimal number of validation controls required for a functional assay to provide supporting or moderate evidence in the absence of rigorous statistical analysis, we estimated the Odds of Pathogenicity (OddsPath) that could be obtained from evaluating various numbers of previously classified controls (Additional file 1). This was done by assuming that the assay readout would be indeterminate for about 20% of variants, the remaining known pathogenic controls would have a functionally abnormal readout, and the remaining known benign variants would have a functionally normal readout. We treated the proportion of pathogenic variants in the overall modeled data as a prior probability (P_1), and the proportion of pathogenic variants in the groups with functionally abnormal or functionally normal readouts as posterior probabilities (P_2). To circumvent posterior probabilities of zero or infinity, we added exactly one misclassified variant to each set [13]. The OddsPath was then estimated for each as: OddsPath=$[P_2 \times (1- P_1)] / [(1- P_2) \times P_1]$ [14].

POINTS TO CONSIDER AND GENERAL RECOMMENDATIONS

Physiologic context

The genetic construct and context being evaluated in an assay are important considerations for determining appropriateness for clinical variant interpretation. The assay material being utilized (e.g., patient-derived sample, model organism, cellular *in vivo* or *in vitro* system) should be taken into account when evaluating the validity of a functional assay. When using patient-derived samples, a functional assay evaluates a broader genetic and physiologic background (other variants *in cis* and *in trans*, epigenetic effects, cell type, assay conditions, etc.). For conditions inherited in an autosomal recessive pattern, biallelic variants are required, often in a loss of function mechanism where the penetrance and expressivity of disease

manifestations may depend on thresholds of overall protein activity that reflect the cellular/biochemical phenotype arising from a combination of variants and potentially other cellular gene products. In this case, it will be important to distinguish the overall protein activity levels that cause different phenotypes (severe versus mild disease) from the functional assay results that would qualify for variant-level evidence toward a pathogenic or benign interpretation. If a variant is known to be homozygous (either by segregation analysis or exclusion of a large deletion *in trans*), and can be evaluated in multiple unrelated individuals, functional assay evidence from patient-derived material can be interpreted with greater confidence.

> ***Recommendation 1:*** Functional evidence from patient-derived material best reflects the organismal phenotype and, in general, it would be better to use this evidence to satisfy PP4 (specific phenotype) and to delineate the expected disease phenotype in patients with certain combinations of variants or homozygous variants with known pathogenicity. If the VCEP decides to proceed with evaluating an assay performed in patient-derived material, the level of strength applied should be determined based on validation parameters (see below). In addition, the VCEP should specify the required number of unrelated individuals in whom the variant has been tested, in order for the evidence to qualify for variant interpretation.

Typically, model organisms are used to implicate the role of a gene in a disease (e.g., the gene is deleted, interrupted, or an artificial mutation is made to recapitulate a phenotype as evidence of the genetic etiology). Issues related to cost and throughput have typically limited the generation of extensive allelic series intended for the purpose of clinical variant interpretation. In

addition, it can be challenging to assess how well the model organism reflects a human anatomy/physiology/genetic context, or whether the full phenotype must necessarily be recapitulated in order to satisfy the functional evidence criteria. The genome of the organism may include an orthologous gene (having equivalent or similar function), or the model organism may lack relevant homologs that affect the phenotype in humans, thus affecting the degree to which an artificially introduced genetic variant can cause a relevant phenotype. Even within a given species, measurable phenotypes can vary depending on the genetic background of the organism (e.g., compensatory variation) and therefore studies using more than one strain or line would be preferable, further increasing the cost of such assays. Therefore, the recommendations herein will primarily focus on cellular and biochemical *in vivo* or *in vitro* assays, which are commonly encountered in laboratory evaluations of variants implicated in human disease.

> ***Recommendation 2:*** From the point of view of clinical variant interpretation, evaluation of functional evidence from model organisms should take a nuanced approach, considering the caveats described above. If model organism data are to be used in variant interpretation, the strength of evidence should be adjusted based on the rigor and reproducibility of the overall data provided.

Molecular consequence

The nature of the variant and the context in which it is studied can significantly affect the assay readout. The effect of the variant on the expressed gene product must be carefully considered when determining the clinical validity of an assay that utilizes an artificially engineered variant. For example, CRISPR-introduced genetic variants in an otherwise normal genomic context will use the endogenous cellular transcriptional and splicing machinery,

although off-target effects must be carefully considered. In contrast, transient expression of cDNA constructs, which usually contain artificial promoters and other regulatory sequences that can result in variant overexpression, should be carefully standardized using controls to ensure that the overexpression does not mask the true effects of variants. Nonsense and frameshift variants that result in premature termination codons before the 3'-most 50 nucleotides of the penultimate exon are expected to undergo nonsense-mediated decay and eliminate the mRNAs [15,16]; therefore, studying such variants in the context of cDNA may not reflect the endogenous situation. Similarly, the effects of a nucleotide substitution on splicing cannot be assessed using a cDNA construct. On the other hand, when the variant results in an expressed protein with in-frame deletion or single nucleotide substitution, an engineered cDNA construct may reasonably reflect the functional impact, at least at the protein level.

> ***Recommendation 3:*** While testing variants in a more natural genomic context is preferable, it is not a requirement of a well-validated assay. Instead, one should consider how the approach impacts the interpretation of the results and take into account whether the study controls for these limitations when assigning strength of evidence.

Since an individual functional assay may not fully capture all gene or protein functions relevant to disease pathogenesis, a "normal" result in a laboratory assay may simply reflect that the functional effect of the specific variant was not suitably assayed in the experiment. Therefore, in order to determine when, and at what strength, to apply the BS3 criterion, it is essential to understand how well the assay captures the molecular consequence of the variant and its impact on the expressed protein or functional domain. A more complete assessment of protein function permits scoring the result as having a benign effect, whereas an assay that is limited to a

specific domain or functional readout may provide less strong evidence for having a benign effect.

Messenger RNA splicing is a complex process, and clinical variant interpretation can take into account both predictive and laboratory evidence. RNA splicing assays, developed using the endogenous genomic context, or using artificial mini-gene assays, can be useful to determine the impact of variants on splicing integrity. However, unlike protein assays, the readout (e.g., exon skipping, or intron retention) does not necessarily correlate with protein function. For example, abnormal splicing of the last exon might lead to a truncated protein whose function is still intact. In general, abnormal splicing can have heterogenous outcomes with respect to mRNA fate and the protein-reading frame. Abnormally spliced transcripts might undergo nonsense-mediated decay, while other abnormal transcripts can lead to a shortened or truncated protein with or without functional consequences [17]. Finally, it should be noted that a missense or synonymous variant that does not affect protein function or level might have a negative impact on splicing.

Recommendation 4: Because RNA splicing assays do not provide a direct measure of protein function, additional recommendations are needed to determine the applicability of splicing assays to satisfy PS3/BS3 versus PVS1 (loss-of-function). For canonical ±1,2 splice variants, splicing assays could be used to refine the application and enhance strength of the PVS1 criterion, where currently the predicted impact of a variant on mRNA stability and protein-reading frame is used for this assessment [13]. For variants outside the canonical ±1,2 splice sites, an SVI splicing subgroup is working on recommendations for including evidence of predicted altered splicing into the

ACMG/AMP framework. For variants in which the splicing assay shows an impact that is not predicted to lead to loss-of-function (such as in-frame exon skipping or large in-frame deletions), PS3/BS3 could still be appropriate if the assay examines the protein function of the resulting product.

Terminology

Standardized, structured language can improve communication and transparency across clinical laboratories, physicians, and patients. Uniform terminology should be used to describe the readout of a laboratory assay of protein function and document the curation of functional evidence. As such, the variant-level results of functional assays should not be categorized as "pathogenic" or "benign," since these falsely equate functional impact with a determination that involves a number of other evidence lines. In addition, terms describing assay results as "deleterious", or "damaging" can be confusing since their meanings are greatly context-dependent and generally only apply when loss of function is the mechanism of disease. For example, in conditions where the mechanism involves gain of function, a variant may be damaging or deleterious to the organism but not to protein activity as measured in a functional assay.

Recommendation 5: The terms "functionally normal" or "functionally abnormal" should be used to describe the functional impact of a variant as measured in a given assay. Further granular specifications should be used to describe the "functionally abnormal" impact (i.e., complete loss of function, partial loss of function/intermediate effect/hypomorphic, gain of function, dominant-negative) as outlined by Spurdle, et al.

[18]. The final assessment of the evidence should take into account both the functional effect in the assay and the mechanism of disease (see below).

CLIA laboratory developed tests

The 2015 ACMG/AMP guidelines assert that "functional studies that have been validated and shown to be reproducible and robust in a clinical diagnostic laboratory setting are considered to be the most well established" [1]. All tests conducted in a Clinical Laboratory Improvement Amendments (CLIA) laboratory or with a commercially available kit are subject to analytical validation for in-house use. However, these assays should also be evaluated for the strength of evidence based on the controls used, as detailed below. One should also consider that *in vitro* assays developed in CLIA laboratories that are conducted with patient samples for diagnostic use [19] may not necessarily provide variant-level evidence relevant to interpretation (see Recommendation 1, above). Data from research laboratories are not subjected to specific regulatory oversight and thus may be validated to different degrees, though any *in vivo* or *in vitro* study can satisfy PS3/BS3 criteria with a strong level of evidence if it demonstrates the appropriate validation.

Recommendation 6: The entity performing a functional assay should not govern whether PS3/BS3 criteria are satisfied, or at what strength. This determination should be based primarily on the validation of the assay, including the use of appropriate laboratory controls as well as clinical validation controls (as described below).

Experimental controls and clinical validation controls

Good laboratory practice is essential for application of functional evidence in clinical variant interpretation. Every experiment should include internal controls that demonstrate the dynamic range of the assay (e.g., the readout of the assay with wild type and null effect). In some cases, the readout may be normalized to a wild type value, which should generally be run in the same conditions as the variants being tested to avoid a batch effect. Well-conducted experiments typically use technical replicates that control for the random differences associated with protocol or instrument-associated variation, to demonstrate reproducibility of the result within a given experiment. Similarly, biological replicates (e.g., different colonies, cells, aliquots, or animals) are included to control for random biological variation in parallel measures of unique biological samples and to demonstrate reproducibility of the result between instances of the same experiment. Biological replicates are more important for understanding the variance within a population, while technical replicates can reduce measurement error [20].

Furthermore, well-validated assays are benchmarked by including known pathogenic and known benign variants that establish the ranges of assay readout for these classes of variants and define the thresholds beyond which the result can be considered functionally abnormal, indeterminate, or functionally normal. It is important to note that the clinical interpretation of these validation control variants should reach a pathogenic or benign interpretation using lines of evidence independent of functional data, so as to avoid circularity in defining the assay's predictive value. The number of controls required depends on the dynamic range of the assay and the variance of each replicate; controls should also be relevant to the disease mechanism and the type of variant under consideration (e.g., missense controls for evaluating missense variants of uncertain significance). Variants in the Genome Aggregation Database (gnomAD)[21] that have population allele frequencies exceeding the threshold for BA1 or BS1, but have not yet been

added to ClinVar (Clinical Variant Database), could serve as a source of benign controls. Additionally, for studies of missense VUS in these genes, one could consider if pathogenic or benign controls from different genes related via a disease mechanism and functional pathway could be used at a lesser strength of evidence.

FUNCTIONAL EVIDENCE EVALUTION AND APPLICATION

The SVI Working Group recommends that evaluators use a four-step process to determine the applicability and strength of evidence of functional assays for use in clinical variant interpretation: 1. Define the disease mechanism; 2. Evaluate general classes of assays used in the field; 3. Evaluate validity of specific instances of assays; 4. Apply evidence to individual variant interpretation. Unlike the ACMG/AMP guidelines [1], in which well-established functional studies can provide a default "strong" level of evidence (PS3/BS3), the SVI recommends that evaluation of functional assays should start from the assumption of no evidence, and that increasing clinical validation can allow application of evidence in favor of a pathogenic or benign interpretation at a level of strength (supporting, moderate, strong) concomitant with the demonstrated validation metrics as described below.

1. Define the disease mechanism

In order for functional assays to be useful in clinical variant interpretation, the underlying gene-disease mechanism must be reasonably well understood. The VCEP or individual interpreting variants in a given gene should first delineate this mechanism to determine what functional assays can be considered applicable. This is an important first step since some genes are associated with different diseases depending on the mechanism (e.g., gain-of-function vs.

loss-of-function). A structured narrative using ontologies or other specific terms can be used to describe the gene-disease mechanism (Text Box 2).

2. Evaluate general classes of assays used in the field

Next, the general types or classes of assays used in the field should be defined and documented, including the model system, experimental method, and functional outcome being evaluated. The defined gene-disease mechanism should guide an evaluation of how well a general class of assay models pathogenesis (e.g., loss of function, gain of function, specific pathway output, etc.). Relative strengths and weaknesses of the model system (e.g., nature of the genetic construct, cellular/organismal context) should be assessed. Disease-specific assertions regarding the appropriateness of animal models compared to cellular *in vivo* models and *in vitro* biochemical systems should be addressed. For cell-based assays, curators should consider whether human or animal cells better represent the disease mechanism. The purpose of this step is to delineate the types of assays that are deemed appropriate (if sufficiently validated) for use in clinical variant interpretation.

- VCEPs should refrain from making blanket statements limiting the general classes of assay that are deemed valid or applicable, and should not cap the strength of evidence based on the class of assay. Instead, the validation metrics (specified below) should dictate the strength of evidence applied.
- In some cases, a VCEP may wish to endorse a particular type of assay that could be used for variant interpretation if developed in the future. In this case, we recommend that such evidence be used no higher than the “supporting” level until the VCEP is able to formally review the quality of the assay.

- The VCEP should assess whether the given class of assay can evaluate all relevant functions of the protein or provide a readout reflective of the disease mechanism as a whole, in order to establish any limitations of the assay with respect to applying evidence in favor of a benign interpretation.

3. Evaluate validity of specific instances of assays

For the general classes of assay that are deemed applicable, the VCEP should next evaluate specific instances of those assays as performed by various groups. Many different laboratories may generate functional evidence using the same general class of assay, but given the differences in the specific methods used and the level of validation provided by each group, evaluation of each individual assay iteration is required before data can be applied in a clinical interpretation. Considerations include basic experimental controls, variant-level validation controls, and statistical analyses used to establish that the assay readout distinguishes pathogenic from benign variants. Rigorous statistical analyses that establish assay readout thresholds defining a functionally abnormal result, indeterminate result, and functionally normal result, are necessary to reach higher levels of evidence. Assays having sufficient numbers of validation controls to calculate positive predictive value or determine the odds of pathogenicity (OddsPath) provide the most robust functional assay evidence [22]. Without this level of clinical validation, the predictive value of the assay is limited. Steps of this evaluation are shown in Figure 1.

- Functional evidence should not be applied in the following scenarios unless the dynamic range of the assay and the thresholds for defining a functionally normal, indeterminate, or functionally abnormal result are extremely well understood:

    - Assays that do not include both negative (normal or wild-type) and positive (abnormal or null) controls.
    - Assays that do not include technical and/or biological replicates.
    - Assays lacking validation controls (known pathogenic and known benign variants).
- Supporting level evidence in favor of pathogenicity (PS3_supporting) or benign interpretation (BS3_supporting) may be applied in the following scenarios:
    - Classes of assays that have been broadly accepted historically, previously validated, or provided as a kit with defined performance characteristics, but where controls and replicates are not documented for the specific instance of the assay.
    - Assays with at least eight total validation controls including a mix of benign and pathogenic variants, but with no formal statistical analyses to assess the ability to distinguish pathogenic from benign variants (see Additional file 1).
- Moderate level evidence in favor of pathogenicity (PS3_moderate) or benign interpretation (BS3_moderate) may be applied in the following scenarios:
    - Assays with at least 12 total validation controls including a mix of benign and pathogenic variants, but no formal statistical analysis used to assess the ability to discriminate between pathogenic and benign variants (see Additional file 1)

- Any level of evidence in favor of pathogenicity may be applied when rigorous statistical analysis enables a formal OddsPath to be calculated, with the strength of evidence correlating to the calculated OddsPath (Table 1).
- Evidence in favor of a benign interpretation up to a strong level (BS3) may be applied when rigorous statistical analysis enables a formal OddsPath to be calculated, with the strength of evidence correlating to the calculated OddsPath (Table 1).

Table 1: Evidence strength equivalent of Odds of Pathogenicity

Odds of Pathogenicity (OddsPath)	Evidence strength equivalent
<0.053	BS3
<0.23	BS3_moderate*
<0.48	BS3_supporting
0.48-2.1	Indeterminate
>2.1	PS3_supporting
>4.3	PS3_moderate
>18.7	PS3
>350	PS3_very_strong

*Since there are no moderate strength benign evidence codes included in the Richards, et al. guidance, moderate level evidence is equivalent to two instances of supporting-level benign evidence

VCEPs should document the specific assay instances that qualify (and why) and the specific assay instances of assays that do not qualify (and why). Documentation should include PMID or other universal reference to the source of the assay evaluated (e.g., DOI), the type of assay readout (qualitative/quantitative) and units, and the range of assay results that qualify for a given strength of evidence according to level of validation as above, and the range in which the assay result is indeterminate with respect to PS3/BS3.

4. Apply evidence to individual variant interpretation

Once the specific instance of an assay has been evaluated as a whole, the results from that assay for a given variant can be applied as evidence in variant interpretation.

- If the assay demonstrates a functionally abnormal result consistent with the mechanism of disease, the PS3 criterion can be applied at a level of strength based on the degree of validation detailed above.
- If the assay demonstrates a functionally normal result, the BS3 criterion can be applied at a level of strength based on the degree of validation detailed above.
- Variants demonstrating an intermediate level of impact on function merit special consideration, as this could be because the assay does not fully reflect the protein function (decreasing strength applied to the assertion), or may provide evidence supporting a hypomorphic or partial loss-of-function effect, such as in a condition with incomplete penetrance and/or less severe expressivity. Consideration of disease mechanism should help guide the appropriate level of strength to be applied for these types of variants.

Stacking Evidence

When multiple functional assay results are available for a single variant (different instances of the same class of assay performed by different laboratories, or multiple lines of evidence from different classes of assay), the evaluator should apply evidence from the assay that is the most well-validated and best measures the disease mechanism.

- For a variant analyzed by multiple assays (belonging to the same or different class):

    - If the results are consistent (both show a functionally abnormal effect, or both show a functionally normal effect), apply PS3/BS3 at the level of strength appropriate for the most well-validated assay.
    - If the results are conflicting, the assay that most closely reflects the disease mechanism and is more well-validated can override the conflicting result of the other and evidence should be applied at the strength indicated by the assay's validation parameters. If the assays are essentially at the same level of validation, conflicting functional evidence should not be used in the interpretation of the variant.

When applying functional evidence in conjunction with available *in silico* and splicing predictors, it may be appropriate to either stack the evidence or allow functional evidence to replace the predictors.

- Carefully consider what each *in silico* tool evaluates to decide how to apply these different types of evidence. Some predictors only consider evolutionary conservation, which provides evidence of the selection for a given amino acid or nucleotide. Others use crystal structure and biophysical predictions to estimate the effect on folding or enzyme function, in which case an *in vitro* assay of enzyme activity should replace the *in-silico* prediction to avoid double counting the evidence.
- For splicing prediction algorithms, a supporting level evidence code (PP3) can be applied; however, if there is laboratory evidence of abnormal splicing or abnormal protein-level function, then the stronger functional evidence should be applied in its place according to forthcoming SVI guidance.

FUTURE CONSIDERATIONS

Bayesian adaptation

As the field moves to develop assays with sufficient controls and validation to permit the calculation of an odds of pathogenicity (OddsPath), more quantitative approaches for stacking evidence and assigning evidence strength may be adopted, as outlined in the Bayesian adaptation of the ACMG/AMP variant interpretation framework [22]. This quantitative method will reconcile conflicting benign and pathogenic evidence, which is common when considering the results of multiple functional assays, and will help reduce the number of VUS. Furthermore, many assays provide continuous quantitative measures of protein function, and converting their numeric readout to a binary PS3/BS3 interpretation can obscure the richness of those data. Using a more quantitative Bayesian system could convert raw data to odds of pathogenicity that more completely capture the assay results. This would be especially useful for hypomorphic variants that have an intermediate effect on normal protein function.

Multiplexed functional assays

While typical functional assays cited as evidence in variant curations analyze relatively few variants [Kanavy, et al., submitted], new multiplexed assays can analyze thousands of variants in a single experiment [23–25]. This kind of increased throughput facilitates the reproducibility, replication, and assay calibration using many definitive pathogenic and benign variant controls. These metrics are required to determine assay sensitivity and specificity, which can then guide the interpretation of assay readout according to thresholds set by known benign and known pathogenic variant performance. Similarly, thresholds could be drawn based on OddsPath to apply different strengths of evidence based on the specific assay result. Multiplexed

assays are still heavily dependent upon the existence of well-characterized pathogenic and benign variants for assay validation. The availability of allelic variant controls may be limited for some genes, but threshold determination may still be feasible depending on the assay's dynamic range and the distribution of results relative to null and wild-type controls (including variants with high allele frequency incompatible with a pathogenic role for rare Mendelian diseases). In the future, these large datasets of functional evidence could be ingested into the Variant Curation Interface (VCI) or Evidence Repository and made available to variant curators in an automated fashion alongside pre-determined thresholds for interpretation and strength assignment, expediting the curation process. Such an automated repository could re-assess sensitivity and specificity automatically as more variants are added. It is important to note that even if the functional data reach an OddsPath equivalent to very strong evidence, the functional evidence criteria are not stand-alone evidence for either a benign or pathogenic classification and at least one other evidence type (e.g., PS4, prevalence in affected individuals is significantly increased relative to controls) is required to reach a pathogenic classification.

Prioritization methods for functional assay development and validation

As it is time-consuming and expensive to develop and sufficiently validate novel functional assays, effort and resources should be directed to have the greatest clinical benefit. One could prioritize assays that would examine genes with the greatest number of genetic tests performed or individuals tested annually, or focus on the genes with the greatest proportion of VUS that could be adjudicated with functional evidence [2]. Alternatively, one could focus on genes associated with highly actionable conditions, where a change in variant interpretation might dramatically change medical management (e.g., *BRCA2* VUS would be reclassified as

likely pathogenic with functional evidence, leading to increased early surveillance and recommendations regarding cancer prophylaxis and management).

We hope that these recommendations will help develop productive partnerships with basic scientists who have developed functional assays that are useful for interrogating the function of a variety of different genes [26]. Realistically, many researchers may not envision a use for their assays in clinical variant interpretation, and may not recognize the need for extensive validation when applying this evidence clinically (nor possess the expertise to independently determine the clinical interpretation of variants in the gene of interest). We look forward to partnerships between VCEPS and basic scientists to apply the results of *in vitro* and *in vivo* tests in clinical variant interpretation. Publishing and/or submitting these results to ClinGen along with appropriate documentation of validation and thresholds for interpretation will greatly enhance curation and application of these data. Greater awareness of the validation requirements, especially the use of an allelic series containing known pathogenic and known benign variants to evaluate the predictive value of the assay, may enable such assays to be used for clinical interpretation more broadly in the future.

LIST OF ABBREVIATIONS

ACMG: American College of Medical Genetics and Genomics; **AMP:** Association for Molecular Pathology; **BA1:** allele frequency data as stand-alone evidence of benign impact; **BS1:** allele frequency greater than expected for disease, strong evidence of benign impact; **BS3:** well-established functional studies provide strong support of a benign effect; **cDNA:** complementary deoxyribonucleic acid; **CLIA:** Clinical Laboratory Improvement Amendments; **ClinGen:** Clinical Genome Resource; **ClinVar:** Clinical Variant Database; **CRISPR:** clustered

regularly interspersed short palindromic repeats; **DOI:** Digital Object Identifier; **gnomAD:** Genome Aggregation Database; **mRNA:** messenger ribonucleic acid; **OddsPath:** odds of pathogenicity; **PMID:** PubMed Identifier; **PP3:** computational, supporting-level evidence of pathogenic impact; **PP4:** phenotype is highly specific for disease, supporting-level evidence for pathogenicity; **PS3:** well-established functional studies providing strong support of a pathogenic effect; **PS4:** prevalence in affected individuals is significantly increased relative to controls, strong evidence of pathogenic impact; **PVS1:** null variant where loss-of-function is a known mechanism of disease, very strong evidence of pathogenicity; **RT-PCR:** real-time polymerase chain reaction; **SVI:** Sequence Variant Interpretation Working Group; **VCEP:** Variant Curation Expert Panel; **VCI:** Variant Curation Interface; **VUS:** variant of uncertain significance;

Declarations

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Authors' contributions

SEB and JSB conceived the study. SVT developed the model and analysis of evidence strength. SEB, JSB, AAT, SMH, SMM, SVT and DMK drafted the manuscript. SEB, JSB, SMM, AAT, SMH, AAT, LMS, CDH, MSG, GC, FJC, XL, and LGB reviewed and edited the manuscript draft. All authors read and approved the final manuscript.

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The opinions and recommendations expressed here are those of the authors and do not necessarily reflect the policies of any institutions or other organizations to which they are affiliated.

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Figure Legends

Figure 1: Decision tree for evaluation of functional data for clinical variant interpretation.

The SVI Working Group recommends that evaluators use a four-step process to determine the applicability and strength of evidence of functional assays for use in clinical variant interpretation (evidence codes PS3/BS3): 1. Define the disease mechanism; 2. Evaluate general classes of assay used in the field; 3. Evaluate validity of specific instances of assays; 4. Apply evidence to individual variant interpretation.

Text Box 1: Text of Original ACMG/AMP Recommendation for Functional Assays, Reproduced with Permission [1]

"Functional studies can be a powerful tool in support of pathogenicity; however, not all functional studies are effective in predicting an impact on a gene or protein function. For example, certain enzymatic assays offer well-established approaches to assess the impact of a missense variant on enzymatic function in a metabolic pathway (e.g., α-galactosidase enzyme function). On the other hand, some functional assays may be less consistent predictors of the effect of variants on protein function. To assess the validity of a functional assay, one must consider how closely the functional assay reflects the biological environment. For example, assaying enzymatic function directly from biopsied tissue from the patient or an animal model provides stronger evidence than expressing the protein *in vitro*. Likewise, evidence is stronger if the assay reflects the full biological function of the protein (e.g., substrate breakdown by an enzyme) compared with only one component of function (e.g., adenosine triphosphate hydrolysis for a protein with additional binding properties). Validation, reproducibility, and robustness data that assess the analytical performance of the assay and account for specimen integrity, which can be affected by the method and time of acquisition, as well as storage and transport, are important

factors to consider. These factors are mitigated in the case of an assay in a Clinical Laboratory Improvement Amendments laboratory–developed test or commercially available kit. Assays that assess the impact of variants at the messenger RNA level can be highly informative when evaluating the effects of variants at splice junctions and within coding sequences and untranslated regions, as well as deeper intronic regions (e.g., messenger RNA stability, processing, or translation). Technical approaches include direct analysis of RNA and/or complementary DNA derivatives and *in vitro* minigene splicing assays."

Text Box 2: Components of the structured narrative describing the gene-disease mechanism.

- Gene name: HUGO Gene Nomenclature Committee (HGNC) gene symbols [27]
- Associated disease: Monarch Disease Ontology (MONDO) terms [28]
- Mode of inheritance: structured MONDO terms
    - Autosomal dominant (HP:0000006)
    - Autosomal recessive (HP:0000007)
    - Mitochondrial (HP:0001427)
    - X-linked (HP:0001417)
    - Undetermined (HP:0000005)
- Molecular mechanism of disease pathogenesis:
    - Loss of function
    - Gain of function
    - Dominant negative
- Biological pathways: Gene Ontology (GO) terms [29,30]

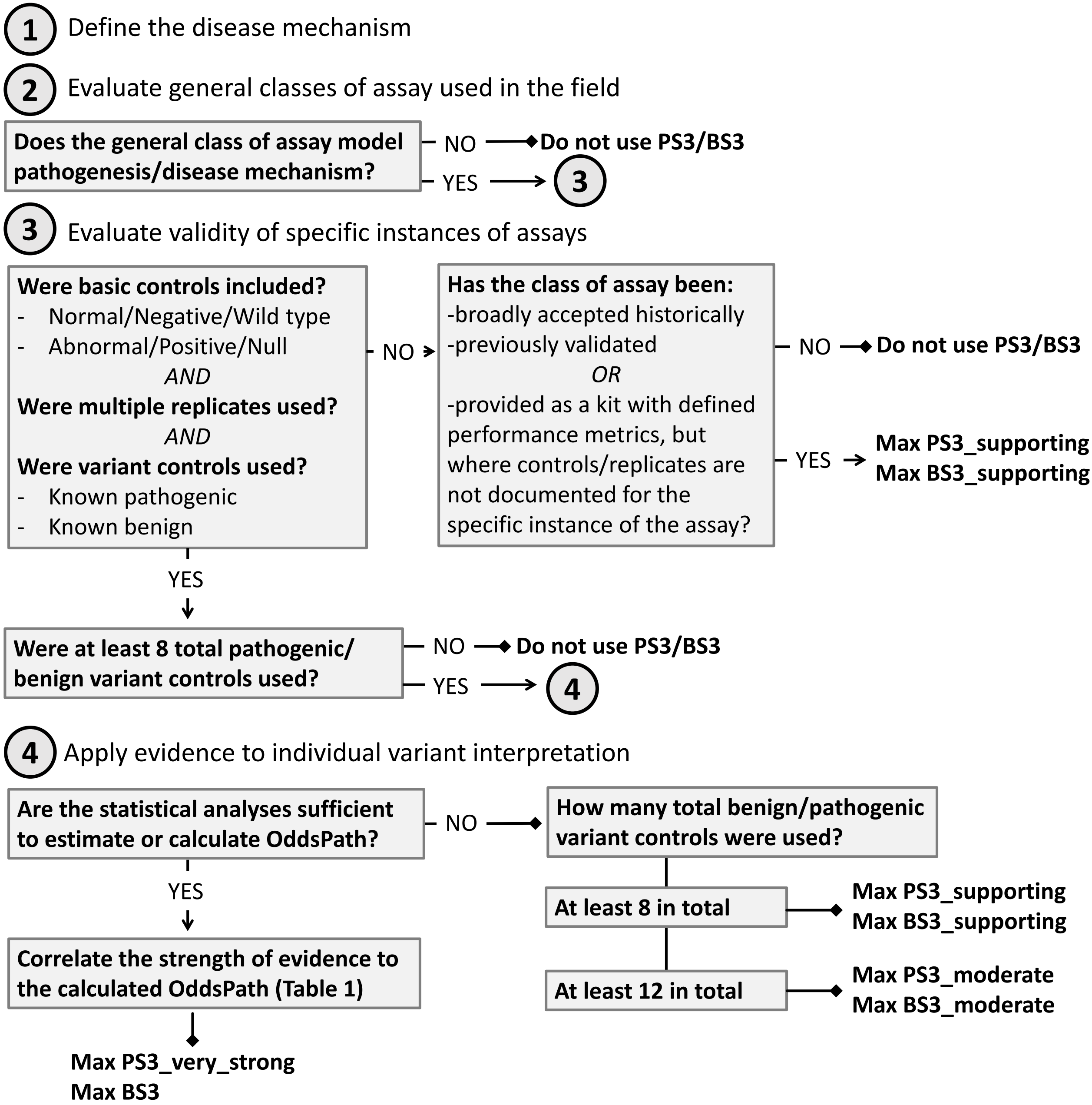
Additional Files:

File name: Additional file 1.xlsx

File format: Excel (.xlsx)

Title of data: Odds of Pathogenicity (Odds Path) by performance of classified variant controls

Description of data: Additional file 1 is a table modeling the Odds of Pathogenicity and strength of evidence based on the number of known benign and known pathogenic variants tested and the ability of the assay to correctly classify those variants.


1 Define the disease mechanism
2 Evaluate general classes of assay used in the field
Does the general class of assay model pathogenesis/disease mechanism?
NO
Do not use PS3/BS3
YES
3
3 Evaluate validity of specific instances of assays
Were basic controls included?
- Normal/Negative/Wild type
- Abnormal/Positive/Null
AND
Were multiple replicates used?
AND
Were variant controls used?
- Known pathogenic
- Known benign
NO
Has the class of assay been:
-broadly accepted historically
-previously validated
OR
-provided as a kit with defined performance metrics, but where controls/replicates are not documented for the specific instance of the assay?
NO
Do not use PS3/BS3
YES
Max PS3_supporting
Max BS3_supporting
YES
Were at least 8 total pathogenic/ benign variant controls used?
NO
Do not use PS3/BS3
YES
4
4 Apply evidence to individual variant interpretation
Are the statistical analyses sufficient to estimate or calculate OddsPath?
NO
How many total benign/pathogenic variant controls were used?
At least 8 in total
Max PS3_supporting
Max BS3_supporting
At least 12 in total
Max PS3_moderate
Max BS3_moderate
YES
Correlate the strength of evidence to the calculated OddsPath (Table 1)
Max PS3_very_strong
Max BS3