An *In Silico* Assessment of Functional Assay Impact on Clinical Variant Interpretation

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Introduction

- Clinical and presymptomatic screening applications of genomic sequencing require additional tools for clinical variant interpretation.
- Variant interpretation guidelines provide a general framework for this process, but significant gaps exist in the ability to utilize evidence such as functional assays.

	← Benign →		← Pathogenic →			
Data Type	**Strong**	**Supporting**	**Supporting**	**Moderate**	**Strong**	**Very strong**
Population	BA1, BS1, BS2			PM2	PS4	
Predicted Effect		BP1, BP3, BP4, BP7	PP3	PM4, PM5	PS1	PVS1
Functional	BS3		PP2	PM1	PS3	
Segregation	BS4		PP1 →			
De novo				PM6	PS2	
Allelic		BP2		PM3		
Database		BP6	PP5			
Other		BP5	PP4			

Figure 1: ACMG/AMP evidence strength by type.[1]

- Many identified genetic variants remain in the category of variant of uncertain significance (VUS) due to insufficient evidence.
- VUS cannot be used for clinical decision-making or risk assessment and thus complicate patient counseling.
- Functional assays are heralded as the solution to the evidence gaps restricting variants to the VUS category, but the impact of functional data on ACMG/AMP classification has not yet been assessed.

Methods

We performed an *in silico* analysis of the ACMG/AMP framework by generating all possible rule combinations (Figure 2) applicable to missense variants. For this purpose, we assumed that each piece of evidence considered was independent and either met/not met.

Pathogenic	(i) 1 Very strong *AND* (a) ≥1 Strong *OR* (b) ≥2 Moderate *OR* (c) 1 Moderate and 1 supporting *OR* (d) ≥2 Supporting (ii) ≥2 Strong *OR* (iii) 1 Strong *AND* (a) ≥3 Moderate *OR* (b) 2 Moderate AND ≥2 supporting *OR* (c) 1 Moderate AND ≥4 supporting
Likely pathogenic	(i) 1 Very strong AND 1 moderate *OR* (ii) 1 Strong AND 1–2 moderate *OR* (iii) 1 Strong AND ≥2 supporting *OR* (iv) ≥3 Moderate *OR* (v) 2 Moderate AND ≥2 supporting *OR* (vi) 1 Moderate AND ≥4 supporting
Likely Benign	(i) 1 Strong and 1 supporting *OR* (ii) ≥2 Supporting
Benign	(i) 1 Stand-alone *OR* (ii) ≥2 Strong
Uncertain significance	(i) Other criteria above are not met *OR* (ii) the criteria for benign & pathogenic are contradictory

Figure 2: ACMG/AMP criteria combining rules for variant interpretation.[2]

- We excluded rules that are not applicable to missense variants (e.g. PVS1 is meant strictly for LOF/ truncating variants)
- We filtered out "unrealistic" combinations (e.g. meeting more than 1 allele frequency criteria)
- Since ACMG/AMP criteria do not provide a method to resolve conflicting VUS, we excluded combinations where conflicting benign and pathogenic criteria exceeded minimal supporting evidence

		Applicability to Variant Type			
Impact	**Evidence**	**Truncating**	**Missense**	**In-frame indel**	**Synonymous**
Pathogenic	PVS1	/	X	X	X
	PS1	/	/	/	X
	PS2	/	/	/	/
	PS3	/	/	/	/
	PS4	/	/	/	/
	PM1	/	/	/	X
	PM2	/	/	/	/
	PM3	/	/	/	/
	PM4	X	X	/	X
	PM5	X	/	X	X
	PM6	/	/	/	/
	PP1	/	/	/	/
	PP2	X	/	X	X
	PP3	/	/	/	/
	PP4	/	/	/	/
	PP5	X	X	X	X
Benign	BA1	/	/	/	/
	BS1	/	/	/	/
	BS2	/	/	/	/
	BS3	/	/	/	/
	BS4	/	/	/	/
	BP1	X	/	X	X
	BP2	/	/	/	/
	BP3	X	X	/	X
	BP4	/	/	/	/
	BP5	/	/	/	/
	BP6	X	X	X	X
	BP7	/	/	/	/

Figure 3: Criteria applicability by variant type

Results

How much would a well-validated functional assay help?

- Focus on VUS due to insufficient information (typically, rare missense variants)
- How could availability of "strong" functional evidence improve the ability to make a pathogenic or benign assertion?

Generate ACMG/ AMP criteria combos, exclude contradictory → Combos that do not include strong functional criteria (PS3/BS3) **476,928** → Examine combos applicable to missense variants **11,730** → Combos classified as non-conflicting "VUS" **3,357**

- Number of classifications that would change with the addition of well-validated, strongly weighted functional data showing a damaging effect or no effect?

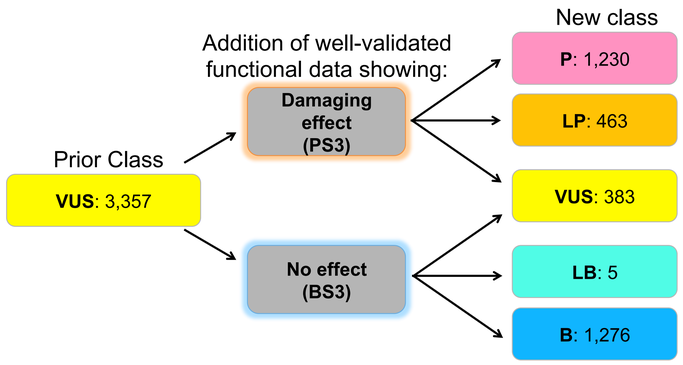

Figure 4: Number of missense VUS rule combinations amenable to reclassification with strong, functional data

- 88.6% of combinations returning VUS due to insufficient evidence could be reclassified with the addition of "strong" functional data

Does strength matter?

- Impact of evidence of lesser strength on clinical interpretation?
- Number of rule combinations that would be reclassified with the addition of "supporting" computational data, "strong" functional criteria, or both?

Generate ACMG/ AMP criteria combos, exclude conflicting → Combos that do not include PS3, BS3, PP3, or BP4 **158,976** → Examine combos applicable to missense variants **4,811** → Combos classified as non-conflicting "VUS" **1,622**

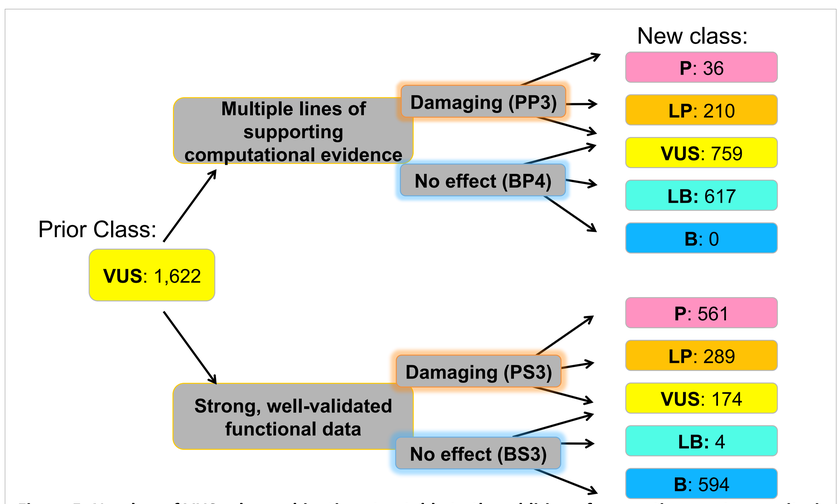

Figure 5: Number of VUS rule combinations tractable to the addition of supporting vs. strong criteria

- 53.2% of these 1,622 missense VUS could be reclassified with supporting-level computational evidence
- 89.3% could be reclassified with strong functional evidence
- 100% could be reclassified with both strong functional evidence & supporting-level computational evidence

- This examines rule combinations tractable to functional evidence, but how many clinically identified variants meet these criteria?

Curated Variant Examples

RASopathy Expert Panel Variant Curation:[3]

- Curated 103 variants total from 9 different genes
- Only 35/103 met PS3, all classified as pathogenic
- 1/103 met BS3, classified as likely benign
- 6 non-conflicting missense VUS
  - 5/6 would be reclassified with PS3 or BS3 criteria

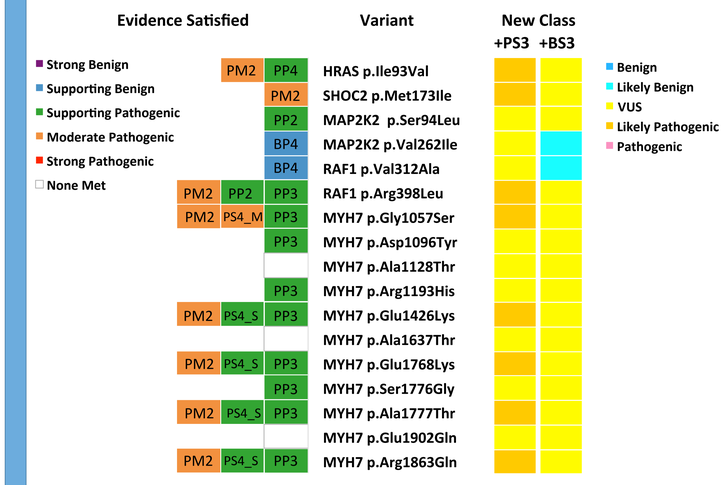

Figure 6: Real-World Impact of Functional Evidence Availability on Expert-Curated Non-Conflicting Missense VUS

***MYH7* –Associated Cardiomyopathies Expert Panel Curation:**[4]

- Curated 60 variants
- Only 4/60 met strong functional criteria, all were pathogenic
- 11 non-conflicting missense VUS
  - 5 would be reclassified as LP with evidence from a well-validated functional assay demonstrating a damaging effect on the gene or gene product
  - 3 only satisfied PP3 criteria and 3 met no criteria for clinical interpretation and thus would remain VUS even with the addition of strong functional evidence

Conclusions

- Well-validated functional assays could improve missense VUS interpretation within the ACMG/AMP framework
- Investigators should focus on developing methods of generating data types that provide "strong" evidence (ex. functional assays)

Future Directions

- Evidence-based prioritization method for assay development and variant assessment
  - Increase capacity for high-throughput functional assay development and variant assessment
  - Reduce the burden of VUS complicating patient care

Acknowledgements & References

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[1] Adapted from Figure 1 in Richards, *et al.*, Interpretation of sequence variants, Genet Med (2015)
[2] Adapted from Table 5 in Richards, *et al.*, Interpretation of sequence variants, Genet Med (2015)
[3] Gelb, *et al.*, ClinGen RAS EP variant interpretation methods, Genet Med (2018)
[4] Kelly, *et al.*, Adaptation of ACMG/AMP variant classification framework for *MYH7*, Genet Med (2018)