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Specifications of the ACMG/AMP guidelines for *ACADVL* variant interpretation:

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Abstract

Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (VLCADD) is a relatively common inborn error of metabolism, but due to difficulty in accurately predicting affected status

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through newborn screening, molecular confirmation of the causative variants by sequencing of the *ACADVL* gene is necessary. Although the ACMG/AMP guidelines have helped standardize variant classification, *ACADVL* variant classification remains disparate due to a phenotype that can be nonspecific, the possibility of variants that produce late-onset disease, and relatively high carrier frequency, amongst other challenges. Therefore, an *ACADVL*-specific variant curation expert panel (VCEP) was created to facilitate the specification of the ACMG/AMP guidelines for VLCADD. We expect these guidelines to help streamline, increase concordance, and expedite the classification of *ACADVL* variants.

Keywords

variant interpretation; pathogenicity; ACADVL; ClinGen

1. Introduction

Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency (VLCADD)(OMIM 201475) is an autosomal recessive disorder caused by biallelic pathogenic variants in the *ACADVL* gene. The VLCAD (EC 1.3.8.8) enzyme, encoded by the *ACADVL* gene, catalyzes the mitochondrial beta-oxidation of long-chain fatty acids with a carbon length chain of 14–20 [1,2]. The frequency of VLCADD is about 1:30,000 to 1:100,000 live births [3,4].

Symptoms of VLCADD include hypoglycemia, cardiomyopathy, hepatopathy, rhabdomyolysis, myoglobinuria, and myopathy and can present in infancy or later in childhood or adulthood. The disease continuum varies from a severe early-onset form with a high incidence of cardiomyopathy and high mortality; an intermediate form with childhood onset, usually with hypoglycemia; and a milder late onset form primarily presenting with myopathy in adulthood. Symptoms can be triggered by prolonged fasting, exercise or fever, and other stressors [5–8].

Treatment includes a low-fat diet, addition of medium chain triglyceride oil (MCT) or triheptanoin, and avoidance of precipitating factors such as prolonged fasting, stress, and dehydration. Standard care for hypoglycemia, rhabdomyolysis, and cardiomyopathy is indicated. Long-term outcomes have improved with early identification and treatment of disease [9–11].

Patients with VLCADD are now typically identified through newborn screening (NBS) by tandem mass-spectrometry analysis of acylcarnitines in dried blood spots. The NBS marker for VLCADD is elevated C14:1-acylcarnitine. Plasma acylcarnitine profile, performed as part of confirmatory tests to follow-up an abnormal newborn screen result, may also reveal the classic profile, but often, the plasma acylcarnitine profile is non-informative, because the patient is metabolically stable. For this reason, follow up testing usually includes molecular analysis. However, not all *ACADVL* variants have been completely curated or investigated to show or prove pathogenicity. In fact, many *ACADVL* variants remain classified as variants of uncertain significance (VUS) [12,13]. For example in Clinvar, a publicly accessible database of classified sequence variants (https://www.ncbi.nlm.nih.gov/

clinvar/) [14,15], there are 499 *ACADVL* VUSs as of February 2023. The presence of so many VUSs confuses and delays diagnosis and therapy. Additionally, biochemical functional analysis of each variant is not commonly available at the clinical level and the enzyme assay in patient cultured skin fibroblasts or blood cells is onerous and may delay the diagnosis [16,17]. More recently, D'Annibale et al [18] have described a microtiter plate assay to study and reclassify inactivating variants expressed in *ACADVL* null HEK293T cell lines.

Harmonization of variant interpretation and classification is a desired step to improve the quality of genetic services across clinical laboratories. In response to an expanding repertoire of sequence variants encountered by massively parallel sequencing, a consensus framework for sequence variant interpretation was endorsed and published by the American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) [19]. Although many laboratories have adopted this framework, inconsistencies in sequence variant classification within and between laboratories have been highlighted [20–23], prompting many to refine and/or expand the framework within the United States and internationally [24,25]. As part of the strategic goals of the Clinical Genome Resource (ClinGen) [26], an expert assessment of gene-level sequence variant interpretation using a standardized approach by convening a Variant Curation Expert Panel (VCEP) [27] can facilitate high quality curation within ClinVar. Additionally, the U.S. Food and Drug Administration (FDA) now recognizes and supports the ClinGen VCEPs as a source of validated information (https://www.fda.gov/media/119313/download). Together, these activities can help facilitate and expedite the clinical reporting of pathogenic variants.

Here we report the work of ClinGen's Metabolism Expert Panel's Acyl-CoA Dehydrogenase Very Long Chain (ACADVL) VCEP, which adapted the ACMG/AMP framework and best practices from ClinGen's Sequence Variant Interpretation (SVI) working group.

2. Methods

The ACADVL VCEP is a subset of the Inborn Errors of Metabolism Clinical Domain Working Group (CDWG) consisting of a diverse team of individuals with expertise in various aspects of clinical genomics and molecular diagnostics, including clinicians, genetic counselors, coordinators, clinical geneticists, and laboratory diagnosticians (https://clinicalgenome.org/affiliation/50048/). A small leadership team consists of two co-chairs and two ClinGen coordinators, serving as a primary point of contact for the group. These individuals represent multiple distinct backgrounds and two different countries, including Canada and the United States. The ACADVL VCEP meets biweekly via teleconferencing software, as well as communicating individually through email, and document sharing websites. Our ACMG/AMP specifications are updated periodically, to find the most current information please visit https://cspec.genome.network.

The ACADVL VCEP assigned small groups of individuals to review each criteria present in the general ACMG/AMP framework and determine whether they were applicable to interpreting variants in *ACADVL* given the associated condition's inheritance pattern, phenotypic data, and prevalence. Each of these groups reviewed the available published data, as well as limited internal data provided by clinicians and group members, and presented

their findings to the VCEP accordingly. Those criteria that were deemed not applicable to *ACADVL* variant interpretation were excluded, while other criteria that required gene or disease-specific modifications were adapted accordingly. Additionally, the general guidance provided by the SVI working group to all VCEPs provided further specifications for certain criteria. An *ACADVL*-specific consideration that was established prior to piloting the codes was the requirement that the variant to be described with HGVS cDNA nomenclature as well as HGVS protein nomenclature as there are publications that utilize protein naming from the processed protein and confusion may occur if the HGVS cDNA nomenclature is not specified.

A collection of 41 variants were chosen as a pilot study based on expert recommendation to determine the VCEP's concordance with published classifications. A team of ClinGen biocurators consisting of both community volunteers and paid employees compiled all published information on each variant and applied the *ACADVL*-specific framework to determine each variant's classification. This information was presented to the VCEP and contrasted to already-published and classifications from external labs, allowing further refinement of the specifications based on the results of these curations. These guidelines were approved by the ClinGen SVI.

3. Results

Existing ACMG/AMP classification guidelines were specified for *ACADVL* based on VLCADD, including determining frequency thresholds, functional assay specifications, and specificity of the disease presentation, as well as modifying the strength for several criteria (Table 1).

3.1 BA1/BS1/PM2

BA1 and BS1 are codes utilizing large population databases to determine if a variant is too frequent to be plausibly pathogenic. PM2 is a population code used to determine if a variant is sufficiently rare that it could plausibly be pathogenic. The strength of PM2 was originally intended to be used as a moderate strength, but based on SVI guidance, the ACADVL VCEP utilized the evidence at the supporting strength (PM2_Supporting, https://clinicalgenome.org/docs/pm2-recommendation-for-absence-rarity/). To determine the allele frequency cutoffs for BA1, BS1, and PM2, we utilized an allele frequency calculator (https://cardiodb.org/allelefrequencyapp/) and used the estimated prevalence of VLCADD of 1:30,000 to 1:100,000 births [28]. We used 0.75 for penetrance to account for mild or late onset VLCADD that may develop at adulthood. The maximum genetic contribution was set to 1 as all cases of VLCADD are caused by pathogenic variants in *ACADVL*. The BA1 threshold was calculated conservatively by utilizing a prevalence of 1:30,000 and a maximum allelic contribution of 1; this results in a conservative allele frequency estimate of greater than or equal to 0.007 (0.7%) for application of BA1 (Table 2). The BS1 threshold was also calculated conservatively utilizing a prevalence of 1:30,000 and a maximum allelic contribution of 0.5, which results in an allele frequency of greater than or equal to 0.0035 (0.35%) for application of BS1. The most common pathogenic variant, c.848T>C (p.Val283Ala), accounts for approximately 20% of all pathogenic alleles, which

helped guide the maximum allelic contribution for calculating PM2 [29]. The maximum credible population allele frequency was calculated considering a prevalence of 1:100,000 for PM2, maximum allelic contribution of 0.2, with maximum genetic contribution and penetrance remaining static as previously established, which results in a frequency of 0.0007 (0.07%), and the ACADVL VCEP multiplied this by 1.5 to account for mildly pathogenic variants being present in carriers within the population databases and reached a cut off of 0.1% for PM2. Therefore, variants with a highest population minor allele frequency (MAF) <0.001 (0.1%) in any continental population with >2000 alleles in gnomAD will meet PM2_Supporting.

3.2 PP3/BP4

For utilizing *in silico* predictors, we recommend the meta-aggregator REVEL to avoid relying on any one specific computational tool or method [30]. Additionally, REVEL is easily available to any curator utilizing the ClinGen Variant Curation Interface (VCI) and other variant curation platforms. To determine our score thresholds for PP3/BP4, we took into account the known scores for *ACADVL* Pathogenic (P)/Likely Pathogenic (LP) and Benign (B)/Likely Benign (LB) variants in ClinVar. Over 80% of the collected P/LP variants had a REVEL score ≥0.75 and the majority of B/LB variants had a REVEL score ≤0.5 (Figure 1), although this analysis was impeded by the low number of B/LB missense *ACADVL* variants. Based on this analysis, we set the PP3 threshold for missense variants at ≥0.75 and the BP4 threshold for missense variants at ≤0.5. These thresholds are concordant with those established by other VCEPs (https://cspec.genome.network/cspec/ui/svi/) [32–34]. In-frame deletions and insertions are not supported by many of the predictors in REVEL, so for these variants, PROVEAN and MutationTaster are used instead. Predictions from these two tools must be concordant to invoke PP3/BP4 for in-frame deletions and insertions.

For splicing predictors we recommend a combination of SpliceAI, MaxEntScan, and NNSplice. This criteria can only be applied if the variant does not meet criteria for PVS1, in order to prevent counting the same type of evidence multiple times. All three of these predictors are publicly available and easily accessible through the ClinGen VCI and other variant curation platforms. PP3 can be applied if the variant meets two of three of the following thresholds: a SpliceAI “high score” (Δ Score ≥0.5 “confidently predicted splice variants”), >15% reduction using MaxEntScan, or >5% reduction using NNSplice. If a new splice-site is predicted to be created, PP3 can be applied if the newly generated splice site is significantly stronger than the wild type site (Δ Score ≥ 0.5 using SpliceAI; >15% difference using MaxEntScan). BP4 can be applied if the variant meets two of three of the following thresholds: a SpliceAI Δ Score ≤0.2, <10% reduction using MaxEntScan, or <2% reduction using NNSplice. These guidelines are consistent with developer-recommended thresholds [35–37].

3.3 PVS1

According to SVI Guidance, *ACADVL* fulfills the three criteria to be eligible to apply PVS1: It has a definitive association with VLCADD, >3 loss of function (LoF) variants are classified as pathogenic without the use of PVS1, and LoF variants make up >10% of the

known variation in this gene. As such, we customized the guidance of the SVI according to the precise structure of *ACADVL* (Figure 2) [38].

The C-terminus region of *ACADVL* is not known to be essential for VLCAD function, therefore we only utilize PVS1_moderate for any variant that is not predicted to undergo nonsense-mediated decay. Any variants that result in a predicted in-frame consequence (such as splice dinucleotide variants or gross exon deletions) are also classified as PVS1_moderate unless predicted to disrupt a region critical to protein function, in which case this can be upgraded to PVS1_strong. There are no known biologically-relevant alternate transcripts for *ACADVL* that would impact interpretation or utilization of this criteria.

3.4 PM1

Mutational hot spots and well-established functional domains can be included at the moderate strength level. For the ACADVL VCEP, critical well-established functional domains were defined and utilized based on the known function of the protein as well as basic research defining the domains. These domains included nucleotide and substrate binding sites from amino acids 214–223, 249–251, 460–466, and 562, membrane binding domain from amino acids 481–516, and the mitochondrial signal peptide from amino acids 1–40 [39,40]. Additionally, the CpG dinucleotides at arginine 326 and 429 have been defined as mutational hotspots and therefore meet PM1 [41].

3.5 BS3/PS3

The ClinGen SVI recommendations for utilizing functional evidence was published as the ACADVL VCEP was establishing these specifications [42]. The VCEP adapted these recommendations for *ACADVL* by requiring functional evidence be generated in non-patient derived material expressing a single *ACADVL* variant while testing of patient-derived material can be considered under PP4. The VCEP uses the validation parameters established in Brnich et al. to set the strength level of the functional evidence and established that if a VLCAD enzyme assay shows ≥20% activity, PS3 can only be utilized at the supporting level. The ACADVL VCEP also established that splicing assays of variants in non-canonical splice sites can be used as PS3 evidence if there is no evidence of normal splicing, taking into account the impact of the splice defect in creating an in frame or out of frame product.

3.6 PP4

Application of the PP4 criterion provides strength of evidence based on individuals that meet a specific phenotype. This can present difficulties for VLCADD as the overt phenotypes, such as hypoglycemia, cardiomyopathy, rhabdomyolysis, and myopathy, are easily conflated with other disorders such as primary muscular dystrophies and cardiomyopathies. Further elevating the difficulty of distinction is that VLCADD has a wide spectrum of severity, which combined with the nonspecific nature of the clinical findings can make phenotypic identification difficult. As such, the general method of diagnosis for VLCADD is biochemical and genetic testing rather than presence of physical features. Dried blood spot acylcarnitine analysis performed during NBS is an important first-line test for VLCADD, with levels of C14:1 being the specific marker for the condition. However, even this results

in a high number of false positive results [43]. Therefore, the results based on NBS alone are not enough to be considered “highly specific” to VLCADD. Specialized biochemical testing, such as confirmatory plasma acylcarnitine profiles and direct analysis of VLCAD enzyme activity in fibroblasts or leukocytes, is widely accepted to clarify the diagnosis of VLCADD. While the former method is less specific, it is also far more clinically available than the specific VLCAD enzyme activity method. Therefore, when specifying this criteria, the general availability and specificity of clinical data was taken into account.

Different strength levels of PP4 can be applied based on the specificity of the biochemical results for any given affected individual that harbors the variant in question. The base level, PP4_Supporting, can be applied if at least one individual has a clinical assertion of reduced VLCAD enzyme activity, enzyme activity 21–27% of normal, NBS C14:1 levels >0.8 μM, or the combination of abnormal NBS “consistent with VLCADD” without specific levels and follow-up plasma acylcarnitine analysis “consistent with VLCADD” without specific levels. PP4_moderate requires a higher threshold and can only be applied if at least one individual has enzyme activity ≤20% of normal or a combination of NBS C14:1 levels ≥1.0 μM with one of the following: enzyme activity 21–27% of normal, abnormal NBS “consistent with VLCADD” without specific levels, or follow-up plasma acylcarnitine analysis “consistent with VLCADD” without specific levels. To establish C14:1 cutoff values the working group reviewed the relevant literature including longitudinal multicenter NBS studies reporting population statistics for C14:1 levels as well as targeted studies reporting NBS findings in symptomatic VLCADD cases. Individuals with C14:1 values greater than or equal to 0.8 uM were found to be very rare in the general NBS population with a frequency of less than 1 in 10,000 whereas the majority of symptomatic VLCAD cases had a C14:1 level exceeding this threshold [44–47]. Therefore, a C14:1 threshold of greater than or equal to 0.8 uM was established as the criteria to invoke PP4_Supporting. Higher C14:1 levels provide even greater specificity for VLCADD detection but with a concomitant reduction in sensitivity. Considering these tradeoffs, a C14:1 threshold of greater than or equal to 1 uM was determined appropriate to invoke PP4_moderate. At this level, the positive predictive value for VLCAD detection has been reported to be 54% [46].

3.7 PP1/BS4/PM3

PP1 (segregation in affected family members) is utilized following the guidance developed by the Hearing Loss VCEP in conjunction with the SVI, which takes into account the logarithm of the odds (LOD) score and number of affected and unaffected segregations to determine if PP1 can be used at the supporting, moderate, or strong level [48].
BS4 (lack of segregation in affected family members) is utilized as established. Briefly, affected segregations and unaffected segregations are considered to calculate the LOD score. Affected segregations are affected individuals in a family, typically consisting of siblings, who harbor the variant in question and an additional variant and is calculated in the LOD by using the number of affected individuals that carry the variant minus 1. To utilize PP1 at a supporting level, the LOD score must be ≥0.6, for the moderate level the score must be ≥1.2 and for strong, the score must be ≥1.5. For utilization of PM3 (detected in *trans* to a pathogenic variant), the ACADVL VCEP utilized SVI guidance for points per proband

without considering uncertain variants detected in *trans* (https://clinicalgenome.org/docs/pm3-recommendation-for-in-trans-criterion-pm3-version-1.0/). This guidance considers the number of probands who carry a pathogenic or likely pathogenic variant confirmed in *trans* by either parental testing or cloning assays at a greater point value, while suspected in *trans* or homozygous occurrence is considered at a lower point value.

3.8 Codes not used

PS2 (*de novo* variant with paternity confirmed in an affected patient without family history) was not used as *de novo* variants in *ACADVL* are very rare but the possibility of de novo variation cannot be excluded and can therefore be considered under PM6. The maximum contribution of a de novo variant, even with maternity and paternity confirmed, can only be used at a moderate level. PS4 (prevalence in affected individuals versus controls) was not used due to a paucity of case controlled studies for VLCADD. BS2 (observed in the homozygous state in a healthy adult) was not used as there are mildly pathogenic variants that may have a difficult to diagnose adult-onset phenotype and individuals homozygous for such variants cannot be considered unaffected. BP5 (variant found in a case with an alternative molecular basis for disease) was not used as an individual could be a carrier of a pathogenic *ACADVL* variant and also be affected with a different disorder. PP2 (missense variant in a gene with few benign missense variants) was not used as there are benign missense variants in *ACADVL*. Similarly, BP1 (missense variant in a gene where loss of function is disease-causing) was not used as both missense and loss of function variants are pathogenic in *ACADVL*. BP3 (in-frame insertions/deletion in a repetitive region without known function) was not used as there are no repetitive regions without known function in *ACADVL*. BP6 and PP5 (reputable source classification) were not used due to ClinGen SVI guidance.

3.9 Piloting *ACADVL*-Specific Criteria

We applied our modified *ACADVL*-specific criteria to a pilot set of 41 variants to test our specifications. The variants were selected from private laboratory data or from the ClinVar database and included variants with previous assertions of benign/likely benign (B/LB), pathogenic/likely pathogenic (P/LP), and variant of uncertain significance (VUS) to allow for wide comparison of how our criteria could be utilized. Case, segregation, and functional evidence were gathered from limited internal data as well as any available published literature. Utilizing these guidelines, 15 variants were assigned a preliminary classification of P/LP, 16 as VUS, and 10 as B/LB. Each of these variants were curated in the ClinGen Variant Curation Interface [49], the classifications were approved by the general ACADVL VCEP, and the classifications submitted to the ClinGen Evidence Repository and ClinVar to be published (Table 3).

Compared to the previous ClinVar classifications, application of these criteria results in an increased number of VUS classifications and a separate increase in B/LB classifications. The elevated VUS rate primarily stems from the more stringent requirements of these criteria compared to the general ACMG/AMP guidelines, more accurately reflecting the lack of published data for these variants. Each of the B/LB classifications had either the

gene-specific BA1 or BS1 population threshold applied, demonstrating that these modified thresholds are essential for downgrading high frequency VUSs to a B/LB classification.

4. Discussion

As with other long-chain fatty acid oxidation disorders (LC-FAOD), VLCADD is associated with considerable clinical, biochemical, and molecular heterogeneity [9,45,50]. More classic or “severe” VLCADD cases tend to have biochemical and clinical features that lead to relatively straightforward diagnosis. However, mild or late-onset cases may be problematic; in some cases, initially abnormal NBS acylcarnitine results resolve upon repeat analysis, molecular testing may be inconclusive, or both [9,46,51]. A particular challenge relates to the inability to distinguish mild VLCADD from heterozygous carriers in some cases [52]. Furthermore, many children with LC-FAOD tend to be asymptomatic as neonates and into early childhood, with disease manifestations developing later in childhood, or even in adolescence or adulthood, so early clinical findings may not be present to help guide the diagnosis [10,44,50,53]. Therefore, following a positive newborn screen, confirmatory testing, primarily involving plasma acylcarnitine analysis and *ACADVL* gene sequencing, is crucial. Functional analyses, such as fibroblast acylcarnitine profiling, immunoblotting, and fibroblast or leukocyte enzyme analysis, may also be helpful if initial biochemical and molecular results are equivocal [9,44,45,54–57].

Improved second-tier NBS assays, such as the incorporation of a metabolomics approach, also have the potential to reduce the number of false positives and improve screening for VLCADD and other disorders [58]. In addition, some relatively clear genotype-phenotype correlations have been established, such as severe phenotypes related to inactivating or null alleles or a late-onset, mild phenotype being typical in those who harbor small in frame deletions/insertions or missense variants that are associated with residual enzyme activity, e.g., c.848T>C (p.Val283Ala), c.1349G>A (p.Arg450His), or c.1820G>C (p.Cys607Ser) [41,44,59–63]. Nevertheless, despite detailed clinical, biochemical, functional, and molecular analyses, it may not be possible to arrive at definitive VLCADD diagnosis in a given case and false negative diagnoses may also occur [64]. Therefore, the continued classification of *ACADVL* variants using the techniques described herein has the potential to provide important data to clinicians caring for potential VLCADD patients, especially those identified by NBS.

Due to ambiguity surrounding interpreting newborn screens, the ACADVL VCEP established specific guidelines for usage of PP4 in regards to the patient’s phenotype. To use PP4 at the supporting level, the NBS C14: levels must be >0.8 μM or an abnormal newborn screen must have follow-up plasma acylcarnitine levels. These minimal criteria are still often difficult to achieve using current literature search alone as often large cohorts of individuals with abnormal newborn screens are published without specifics of individual results or if any follow-up studies were performed. Utilizing biochemical results to apply PP4 is not unique to the ACADVL VCEP, the Phenylketonuria and Mitochondrial VCEPs also utilize laboratory values for application of PP4 while the Cerebral Creatine Deficiency Syndromes, Lysosomal Storage Disorders, and Glanzmann Thrombasthenia VCEPs use a points-based system that considers laboratory abnormalities for application of PP4 (https://

cspec.genome.network). Establishing these specific enzymatic guidelines could be modified for application in other inborn errors of metabolism with a specific biochemical test used as confirmation.

The type of data available to variant curators in the ACADVL VCEP regarding patients identified before NBS is significantly different from information available in the NBS era. Cloning and characterization of the *ACADVL* gene and identification of nine variants in four patients was initially reported in 1996 [41,65]. Enzymatic activity in fibroblasts from patients was used to support the pathogenicity of the variants in addition to several biochemical markers including an abnormal acylcarnitine profile in plasma measured by MS/MS. Additional biochemical findings, although not specific for VLCAD deficiency and not often present, are: dicarboxylic aciduria without glycine conjugates measured by gas chromatography/mass spectrometry (GC/MS) analysis of urine, identification and quantitation of intermediates of unsaturated fatty acid metabolism in plasma by GC/MS analysis, and oxidation of 14C-labeled palmitic acid in intact fibroblasts. As stated above this extensive amount of patient information is not available for the majority of individuals identified by NBS, often making it difficult to reach likely pathogenic/pathogenic classification of a variant.

Given the clear genotype-phenotype correlation that has been shown for *ACADVL* variants, functional assays that reflect the enzyme activity of individual variants are invaluable for interpretation efforts. However, the most widely available functional assays utilize patient-derived lymphocytes or cultured fibroblasts to determine the enzyme activity using dehydrogenation of palmitoyl-CoA [41]. While this is useful for patient diagnostic purposes, it is less useful in the context of single variant interpretation due to potential confounding effects of the other allele. *In vitro* enzyme activities have also been performed, but are comparatively uncommon. Expression systems ranging from COS-7 cells to *E. coli* have been utilized for these purposes [41,56]. These were initially utilized as-is for the functional assay criteria (PS3/BS3), however the ClinGen SVI recommendations required stricter validation parameters to utilize these assays at the strong evidence level due to concerns including consistent experimental environments and lack of proper controls [42]. The difficulty of securing reliable results for single variant enzyme activity is not unique to the ACADVL VCEP, with others such as the Phenylketonuria VCEP requiring mutant enzyme activity studies in mammalian cells to closely resemble the i*n vivo* environment [66]. As such, broader adoption of these single variant expression assays for *ACADVL* and other metabolic genes is warranted and would allow for greater accuracy and precision in variant classification.

Establishing the PM2 cutoff for allele frequency low enough to be consistent with autosomal recessive VLCADD presented difficulties due to an increased carrier frequency for some common *ACADVL* variants associated with disease. Other VCEPs associated with autosomal recessive disorders have established PM2 cutoffs that vary from 0.1% for lysosomal storage disorders (*GAA*), to 0.02% for phenylketonuria (*PAH*), to 0.002% for mitochondrial disorders (*ETHE1*). This can vary even more widely for the benign cutoffs (BA1/BS1), with the Phenylketonuria VCEP utilizing a 1.5%/0.2% threshold and the Lysosomal Storage Disorder VCEP utilizing a 1%/0.5% threshold. The wide range of

frequency cutoffs reflects the value of disease-specific interpretation; accounting for disease prevalence, penetrance, and carrier frequency of variants in each of these specific cases allows for increased accuracy for variant classification compared to general interpretation. Establishing REVEL cutoffs for BP4 also presented a challenge due to the limited number of known benign missense variants. Notably, SVI-recommended thresholds for utilizing REVEL were published after our pilot study was completed [31]. Although our current recommendations for BP4 are in-line with existing expert panels [32–34], we intend to reexamine our REVEL thresholds with a larger dataset to determine how the Pejaver et al publication recommendations impact ACADVL-specific interpretation.

Herein we report the ACMG/AMP guideline specifications for *ACADVL* variant interpretation that have been ClinGen approved. Each criteria was thoroughly evaluated and either amended or excluded from consideration to allow for the complicated aspects of VLCADD. Usage of these guidelines will increase variant classification concordance and will assist in correct diagnoses for patients. All of the ACADVL VCEP variants have been submitted to ClinVar for public usage.

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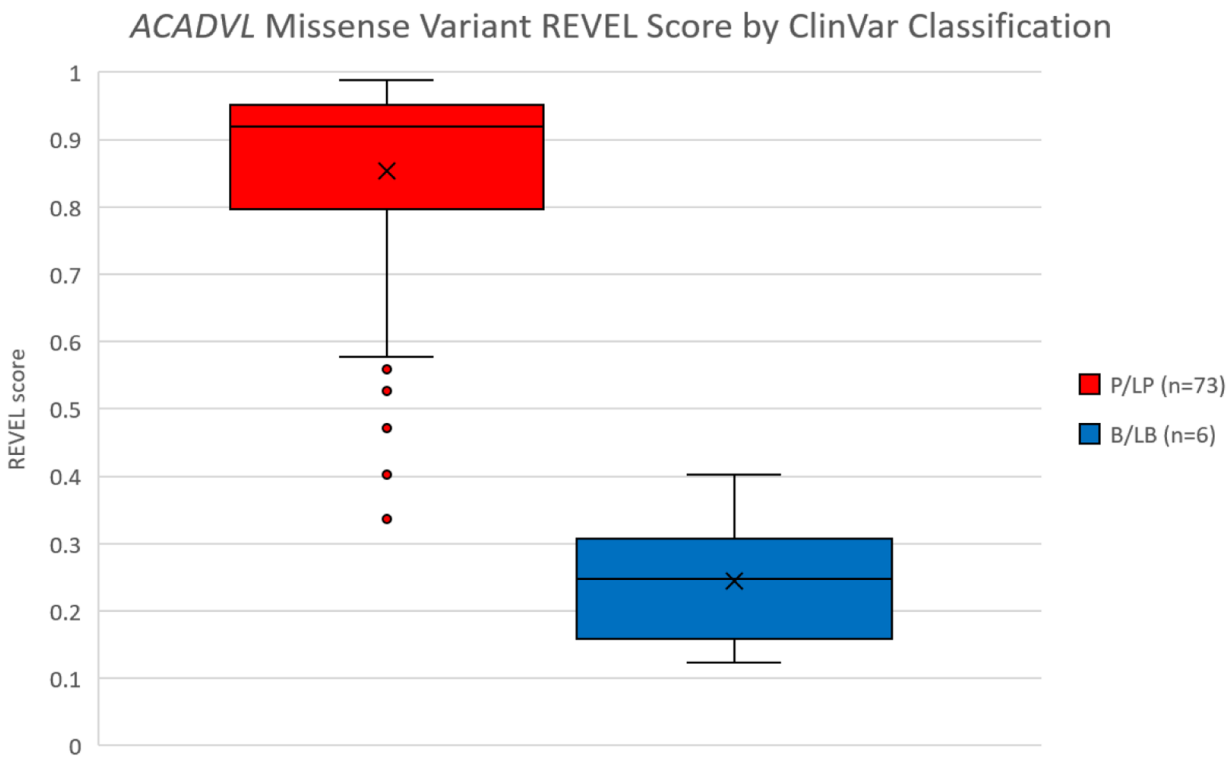

Figure 1.

Comparison of the REVEL score of missense variants to their ClinVar classification. Variants classified as pathogenic (P), likely pathogenic (LP), likely benign (LB), and benign (B) were compared to their REVEL score.

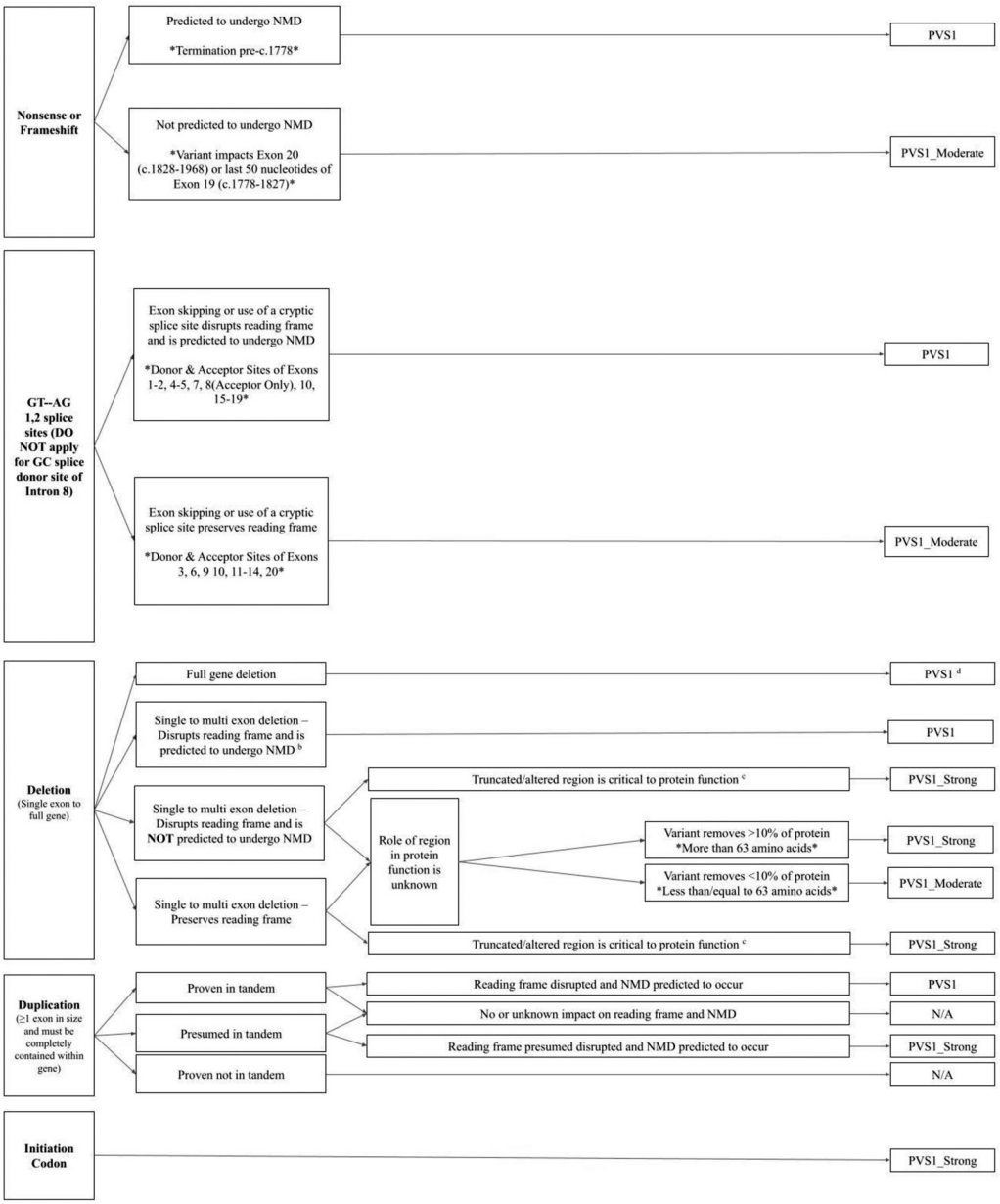

Figure 2: Specification of the PVS1 guideline to the *ACADVL* gene.
This figure was adapted from [38] notations from original publication.

Table 1

Modifications of the ACMG/AMP guidelines for *ACADVL*. Categories marked NA are not used for *ACADVL* classification, categories marked None were not modified. Other criteria were modified to be specific for the disease, to modify the strength of the code, or both.

Pathogenic criteria		
Criteria	**Criteria Description**	**Specification update**
Very Strong Criteria		
PVS1	Null variant in a gene where loss of function is a known mechanism of disease.	Disease-specific, strength
Strong Criteria		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	None
PS2	De novo (paternity confirmed) in a patient with the disease and no family history.	NA
PS3	Well-established in vitro or in vivo functional studies supportive of a damaging effect.	Disease-specific, strength
PS4	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls.	NA
Moderate Criteria		
PM1	Located in a mutational hot spot and/or critical and well-established functional domain.	Disease-specific
PM3	For recessive disorders, detected in trans with a pathogenic variant.	Disease-specific, strength
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.	Disease-specific
PM6	De novo without confirmation of paternity and maternity.	None
Supporting Criteria		
PM2_Supporting	Absent/rare from controls in an ethnically-matched cohort population sample.	Disease-specific, strength
PP1	Co-segregation with disease in multiple affected family members.	Disease-specific, strength
PP2	Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.	NA
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product.	Disease specific
PP4	Phenotype specific for disease with single genetic etiology.	Disease specific, strength
PP5	Reputable source reports the variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation.	NA
Benign criteria		
Criteria	**Criteria Description**	**Specification update**
Stand alone criteria		
BA1	Allele frequency above 5%.	Disease-specific
Strong Criteria		
BS1	Allele frequency greater than expected for disease.	Disease-specific
BS2	Observed in the homozygous state in a healthy adult.	NA
BS3	Well-established in vitro or in vivo functional studies show no damaging effect on protein function.	Disease-specific
BS4	Lack of segregation in affected members of a family.	None
Supporting Criteria		
BP1	Missense variant in a gene where only loss of function causes disease.	NA

BP2	Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in cis with a pathogenic variant in any inheritance pattern.	None
BP3	In-frame deletions/insertions in a repetitive region without a known function.	NA
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product	Disease-specific
BP5	Variant found in a case with an alternative molecular basis for disease.	NA
BP6	Reputable source reports variant as benign but the evidence is not available to perform an independent evaluation.	NA
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

Table 2.

The criteria utilized for specifying the population cut offs for BA1, BS1, and PM2_Supporting.

	gnomAD frequency	Prevalence	Allelic contribution	Genetic contribution	Penetrance
BA1	≥0.0067 (rounded to 0.7%)	1:30,000	1	1	0.75
BS1	≥0.00333 (rounded to 0.35%)	1:30,000	0.5	1	0.75
PM2	〈 0.00073 × 1.5 = 0.1%	1:100,000	0.2	1	0.75

Table 3

Pilot variants, their ClinVar classification, the classification using the ACADVL VCEP modified ACMG/AMP criteria, and the criteria applied. Variants marked as VUS/Conflicting were either classified as variant of uncertain significance or the classifications were conflicting. Variants marked NA were not available in ClinVar at the time of pilot study.

Variant Information	ClinVar Classification	ACADVL Classification	Codes Applied by ACADVL VCEP
NM_000018.4:c.194C>T (p.Pro65Leu)	Benign/Likely Benign	Benign	BA1, BS3_Supporting, BP2, BP4
NM_000018.4:c.49C>T (p.Leu17Phe)	Benign/Likely Benign	Benign	BA1, BP4
NM_000018.4:c.68G>A (p.Arg23Gln)	Benign/Likely Benign	Benign	BA1, BP4
NM_000018.4:c.128G>A (p.Gly43Asp)	Benign/Likely Benign	Benign	BA1, BP2, BP4
NM_000018.4:c.623–8C>T	Benign/Likely Benign	Benign	BA1, BP4
NM_000018.4:c.478–22_478–21delCA	Benign/Likely Benign	Benign	BA1, BP4
NM_000018.4:c.63–35G>A	VUS/Conflicting	Benign	BA1, BP4
NM_000018.4:c.−64T>C	VUS/Conflicting	Benign	BA1, BP4
NM_000018.4:c.1038G>A (p.Ala346=)	VUS/Conflicting	Benign	BA1, BP4, BP7
NM_000018.4:c.1077+15C>T	VUS/Conflicting	Likely Benign	BS1, BP4
NM_000018.4:c.603C>G (p.Tyr201Ter)	Pathogenic/Likely Pathogenic	Likely Pathogenic	PVS1, PM2_Supporting
NM_000018.4:c.1806_1807delCT (p.Leu602_Cys603insTer)	Pathogenic/Likely Pathogenic	Likely Pathogenic	PVS1_moderate, PM2_Supporting, PM3_Supporting, PP1, PP4_moderate
NM_000018.4:c.433C>T (p.Gln145Ter)	Pathogenic/Likely Pathogenic	Likely Pathogenic	PVS1, PM2_Supporting
NM_000018.4:c.192del (p.Lys64fs)	Pathogenic/Likely Pathogenic	Likely Pathogenic	PVS1, PM2_Supporting
NM_000018.4:c.63–2A>C	Pathogenic/Likely Pathogenic	Likely Pathogenic	PVS1, PM2_Supporting
NM_000018.4:c.1077+1G>T	Pathogenic/Likely Pathogenic	Likely Pathogenic	PVS1, PM2_Supporting
NM_000018.4:c.265C>T (p.Pro89Ser)	NA	Likely Pathogenic	PS3_Supporting, PM2_Supporting, PM3, PP3, PP4_moderate
NM_000018.4:c.342+1G>A	NA	Likely Pathogenic	PVS1, PM2_Supporting
NM_000018.4:c.1141_1143delGAG (p.Glu381del)	Pathogenic/Likely Pathogenic	Pathogenic	PM1, PM2_Supporting, PM3_strong, PM4, PP4_moderate
NM_000018.4:c.65C>A (p.Ser22Ter)	Pathogenic/Likely Pathogenic	Pathogenic	PVS1, PS3_Supporting, PM2_Supporting, PM3, PP4_moderate
NM_000018.4:c.753–2A>C	Pathogenic/Likely Pathogenic	Pathogenic	PVS1, PM2_Supporting, PP3
NM_000018.4:c.1077+2T>C	Pathogenic/Likely Pathogenic	Pathogenic	PVS1, PM2_Supporting, PM3_Supporting, PP4_moderate
NM_000018.4:c.277+1G>A	NA	Pathogenic	PVS1, PM2_Supporting, PM3_Supporting, PP4_moderate
NM_000018.4:c.1077+2T>A	NA	Pathogenic	PVS1, PM2_Supporting, PP4_moderate
NM_000018.4:c.103_112dup10 (p.Arg38ProfsTer24)	NA	Pathogenic	PVS1, PM2_Supporting, PM3_Supporting, PP4_moderate
NM_000018.4:c.308A>G (p.Lys103Arg)	Benign/Likely Benign	VUS	PP4_moderate, BA1, BP4
NM_000018.4:c.117C>T (p.Pro39=)	Benign/Likely Benign	VUS	PM2_Supporting, BP7

Variant Information	ClinVar Classification	ACADVL Classification	Codes Applied by ACADVL VCEP
NM_000018.4:c.260T>C (p.Val87Ala)	Pathogenic/Likely Pathogenic	VUS	PM2_Supporting, PM3_Supporting, PP3, PP4
NM_000018.4:c.562G>A (p.Gly188Ser)	Pathogenic/Likely Pathogenic	VUS	PM1_Supporting, PM2_Supporting, PP3
NM_000018.4:c.1066A>G (p.Ile356Val)	VUS/Conflicting	VUS	PP4_moderate, BA1
NM_000018.4:c.477+17G>A	VUS/Conflicting	VUS	PM2_Supporting, BP4
NM_000018.4:c.439C>T (p.Pro147Ser)	VUS/Conflicting	VUS	PM2_Supporting, PM3_Supporting, PP3, PP4
NM_000018.4:c.881G>A (p.Gly294Glu)	VUS/Conflicting	VUS	PM2_Supporting, PM3_Supporting, PP3, PP4_moderate
NM_000018.4:c.1183–7A>G	VUS/Conflicting	VUS	PM2_Supporting, PM3_Supporting, PP3, PP4
NM_000018.4:c.910G>A (p.Ala304Thr)	NA	VUS	PM2_Supporting, PP3
NM_000018.4:c.1966T>A (p.Ter656Arg)	NA	VUS	PM2_Supporting, PM4
NM_000018.4:c.1968A>C (p.Ter656Cys)	NA	VUS	PM2_Supporting, PM4, PP4
NM_000018.4:c.38_49del12 (p.Gln13_Arg16del)	NA	VUS	PM2_Supporting, PM4
NM_000018.4:c.494A>T (p.Glu165Val)	NA	VUS	PM2_Supporting, PP3
NM_000018.4:c.430C>G (p.Leu144Val)	NA	VUS	PM2_Supporting, PP3
NM_000018.4:c.425T>C (p.Phe142Ser)	VUS/Conflicting	VUS	PP3, BS1