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An International Multi-Center Evaluation of Inheritance Patterns, Arrhythmic Risks, and Underlying Mechanisms of CASQ2-Catecholaminergic Polymorphic Ventricular Tachycardia

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Abstract

Background: Genetic variants in calsequestrin-2 (*CASQ2*) cause an autosomal recessive form of catecholaminergic polymorphic ventricular tachycardia (CPVT), though isolated reports have identified arrhythmic phenotypes among heterozygotes. Improved insight into the inheritance patterns, arrhythmic risks, and molecular mechanisms of *CASQ2*-CPVT was sought through an international multi-center collaboration.

Methods: Genotype-phenotype segregation in *CASQ2*-CPVT families was assessed, and the impact of genotype on arrhythmic risk was evaluated using Cox regression models. Putative dominant *CASQ2* missense variants and the established recessive *CASQ2*-p.R33Q variant were evaluated using oligomerization assays and their locations mapped to a recent *CASQ2* filament structure.

Results: A total of 112 individuals, including 36 CPVT probands (24 homozygotes/compound heterozygotes and 12 heterozygotes) and 76 family members possessing at least one presumed pathogenic *CASQ2* variant, were identified. Among *CASQ2* homozygotes and compound heterozygotes, clinical penetrance was 97.1% and 26 of 34 (76.5%) individuals had experienced a potentially fatal arrhythmic event with a median age of onset of 7 years (95% CI: 6–11). Fifty-one of 66 *CASQ2* heterozygous family members had undergone clinical evaluation and 17/51 (33.3%) met diagnostic criteria for CPVT. Relative to *CASQ2* heterozygotes, *CASQ2* homozygote/compound heterozygote genotype status in probands was associated with a 3.2-fold (95% confidence intervals [CI]: 1.3–8.0, $p=0.013$) increased hazard of a composite of cardiac syncope, aborted cardiac arrest, and sudden cardiac death, but a 38.8-fold (95% CI: 5.6–269.1, $p<0.001$) increased hazard in genotype positive family members. *In vitro* turbidity assays revealed that p.R33Q and all 6 candidate dominant *CASQ2* missense variants evaluated exhibited filamentation defects, but only p.R33Q convincingly failed to dimerize. Structural analysis revealed that 3 of these 6 putative dominant negative missense variants localized to an electronegative pocket considered critical for back-to-back binding of dimers.

Conclusions: This international multi-center study of *CASQ2*-CPVT redefines its heritability and confirms that pathogenic heterozygous *CASQ2* variants may manifest with a CPVT phenotype, indicating a need to clinically screen these individuals. A dominant mode of inheritance appears intrinsic to certain missense variants owing to their location and function within the *CASQ2* filament structure.

Keywords

catecholaminergic polymorphic ventricular tachycardia; sudden cardiac death; genetics; arrhythmia; inheritance; structure

Introduction

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmia syndrome characterized by adrenergic-mediated malignant ventricular arrhythmias.^{1–3} CPVT patients often present during childhood with exertion-induced syncope or sudden cardiac death (SCD) in the setting of a structurally normal heart.⁴ Variants in genes whose protein products coordinate calcium-induced calcium release from the cardiac sarcoplasmic reticulum underlie this condition, the most prominent being *RyR2* and *CASQ2*.^{5,6} Whereas *RyR2* gain-of-function variants cause an autosomal dominant form of CPVT, the *CASQ2* genetic subtype is considered autosomal recessive and accounts for 2–5% of CPVT cases.⁷

CASQ2 encodes calsequestrin-2, a high capacity, low affinity calcium binding protein that buffers calcium within the sarcoplasmic reticulum.⁸ High-capacity calcium storage is facilitated by calcium-induced oligomerization of *CASQ2* into filaments that localize to the junctional sarcoplasmic reticulum.^{9,10} Oligomerization, or “filamentation”, is mediated through “front-to-front” dimerization of monomers and subsequent “back-to-back” binding of dimers to form tetramers and higher order polymers that contain calcium-binding sites within electronegative pockets formed at intra-dimer and inter-dimer interfaces.^{11–13} Purported mechanisms for *CASQ2*-CPVT include loss of calcium buffering, altered

regulation of RyR2 activity, and remodeling of the sarcoplasmic ultrastructure and its constituent proteins.^{11,14–19}

Although viewed as an autosomal recessive condition, isolated reports documenting arrhythmias among *CASQ2* heterozygotes have emerged in the literature.^{20–22} A recent X-ray crystallographic structure by members of our group provides novel insight into the *CASQ2* filament structure and reveals that previously reported dominant-acting variants may disrupt back-to-back binding of dimers, while leaving front-to-front dimerization of monomers intact.¹³ Our resulting model proposes that mutant monomers unable to dimerize may be transported out of the sarcoplasmic reticulum and degraded, effectively rescuing a heterozygous phenotype, whereas variants that interfere with the filament while leaving the dimer intact could produce a dominant negative effect.^{13,23–25}

Through an international multi-center collaboration, we sought to further characterize the inheritance patterns and associated arrhythmic risks of *CASQ2*-CPVT. We subsequently pursued *in vitro* functional and *in silico* structural evaluations of candidate dominant *CASQ2* missense variants and the established recessive *CASQ2*-p.R33Q variant in order to glean mechanistic insight into the variable inheritance patterns observed.

Methods

Transparency and Openness Promotion

Data that support the findings of this study are available from the corresponding authors upon reasonable request.

International Multi-Center *CASQ2* (IMCC) Cohort

Probands with a CPVT phenotype presumed secondary to a *CASQ2* variant(s) were identified from 2 international CPVT registries (Amsterdam, Netherlands²⁶ and Vancouver, Canada³; additional details in Online Supplement) and 10 additional centers from North America and Europe. Inclusion into the study required that probands satisfied a CPVT diagnosis consistent with the expert consensus statement²⁷ and possessed at least one *CASQ2* variant classified as pathogenic, likely pathogenic, or as a variant of unknown significance according to criteria from the American College of Medical Genetics and Genomics (ACMG).²⁸ The *CASQ2* variant(s) could be present in a homozygous, compound heterozygous, or heterozygous state. Genotype positive family members were also included. Probands had to have undergone screening of all 105 exons and associated exon-intron boundaries within *RyR2*. Individuals were excluded if they possessed a pathogenic, likely pathogenic or variant of unknown significance in *RyR2* and/or if they carried a pathogenic or likely pathogenic variant in another gene implicated in CPVT or a CPVT compatible phenotype (*TECRL*, *CALM1–3*, *TRDN*, *KCNJ2*, and *ANK2*). Additional exclusion criteria consisted of obstructive coronary artery disease and ventricular cardiomyopathy. Clinical and genetic details recorded for probands and genotype positive family members, including definitions utilized for outcome events, are provided in the Online Supplement. Special care was taken to ensure that no study participants were included twice.

The study was performed as part of a protocol approved by the research ethics boards of Western University, London, Ontario, Canada and the collaborating institutions and all study participants provided informed consent.

Systematic Literature Review of Reported Cases

A comprehensive literature search was conducted using the search terms “CASQ2”, “Calsequestrin-2”, “catecholaminergic polymorphic ventricular tachycardia”, and/or “CPVT” in the MEDLINE electronic database. All reports published before November 2019 in the English language were reviewed if they reported a *CASQ2* variant in association with a CPVT phenotype. Clinical and genetic details, as described for the IMCC cohort, were extracted for all phenotype positive *CASQ2* heterozygotes not already included in our study.

In Silico Evaluation of Pathogenic CASQ2 Variants

All identified *CASQ2* variants from the IMCC cohort and the systematic literature review were subjected to *in silico* prediction of variant deleteriousness and variant classification according to ACMG guidelines.²⁸ Their prevalence was assessed using the Genome Aggregation Database (gnomAD), a repository including over 140,000 individuals from multiple population-based and disease-specific genetic cohort studies.²⁹ *In silico* prediction was performed using Polymorphism Phenotyping v2 (PolyPhen-2), Sorting Intolerant From Tolerant (SIFT), and Combined Annotation Dependent Depletion (CADD).^{30–32}

Cloning of Protein Expression Constructs and Expression and Purification of Cardiac Calsequestrin

Candidate dominant *CASQ2* missense variants from the IMCC cohort and an established recessive variant, CASQ2-p.R33Q, underwent functional evaluation. Calsequestrin clones lacking the signal peptide sequence were ligated using Gibson Assembly into a T7-based bacterial overexpression vector (pET28a) with an upstream 6His site and TEV protease cleavage sequence. Missense mutants were generated using the protocol from the Q5 Site-Directed Mutagenesis Kit (New England BioLabs), using either the Q5 or Phusion polymerases. All constructs were verified by Sanger sequencing and overexpressed in Rosetta(DE3)pLysS *E. coli*. Primers used for cloning and mutagenesis are provided in Table I in the Supplement.

The protocol for expression and purification of cardiac calsequestrin has been previously reported and full details are provided in the Online Supplement.¹³ Briefly, expression constructs were transformed into Rosetta(DE3)pLysS *E. coli*, grown in culture, and induced with 0.25mM IPTG. Following resuspension of pellets in lysis buffer, clarified supernatants were filtered and calsequestrin-containing fractions were isolated by immobilized metal affinity chromatography using a HisTrap FF column. Pooled protein fractions were treated with TEV protease and dialyzed. Anion exchange polishing was performed using a Mono Q column, protein was eluted in a continuous gradient, and fractions were analysed for purity by SDS-PAGE and A260/280 ratio.

Oligomerization and Structural Analysis

Full details regarding the turbidity assays and size exclusion chromatography (SEC) are provided in the Online Supplement. The turbidity assay, through quantitative evaluation of the cloudiness of a solution, was used to evaluate polymerization. Following addition of CaCl₂ solution to recombinant protein samples, absorbance at 350nm was monitored for 45 minutes. SEC was performed using a Superdex200 Increase 10/300 GL column, elution was performed using 1 column volume of buffer, and absorbance (280nm) of eluted proteins was measured at a flow rate of 0.9 mL/min.

All CASQ2 structure figures were prepared in PyMOL using Protein Data Bank structures 6OWV (cardiac calsequestrin) and 6OWW (cardiac calsequestrin with identification of cation-binding sites by ytterbium anomalous signal).³³ Sequence alignments were created with TEXshade.^{33,34}

Statistical Analysis

Normally distributed continuous variables are presented as means \pm standard deviation and were compared using Student's t-test. Comparison of categorical values was performed using Fisher's exact test. Cox proportional hazards models were used to estimate the associations between genotype and the first presumed primary arrhythmic event (composite of presumed cardiac syncope, appropriate implantable cardioverter-defibrillator [ICD] shock, aborted cardiac arrest [ACA], or SCD with normal autopsy; subsequently referred to as the composite arrhythmic outcome with syncope) and the first definite primary arrhythmic event (composite of appropriate ICD shock, ACA or SCD with normal autopsy; subsequently referred to as the composite arrhythmic outcome without syncope). As CPVT imparts risk of arrhythmic events throughout life, the time interval considered for Cox models extended from birth to date of last follow-up. Genotype was dichotomized as heterozygous or homozygous/compound heterozygous for *CASQ2* variants. A robust covariance matrix estimator involving robust standard errors clustered by family was used to account for familial relatedness. Log-minus-log plots and the Schoenfeld test confirmed that all Cox regression models satisfied the proportional hazards assumption for the *CASQ2* genotype covariate.

For turbidity assays, data points in figures represent mean values, with error bars representing standard deviation. All turbidity assay data points are mean of 3 technical replicates.

Two-tailed p-values < 0.05 were considered statistically significant and statistical analyses were performed using Stata version 15 (College Station, TX, USA).

Results

International Multi-Center CASQ2 (IMCC) Cohort

Screening of 2 international CPVT registries and 10 inherited arrhythmia clinics from North America and Europe identified a total of 112 individuals from 36 CPVT families with a presumed culprit *CASQ2* variant, considered pathogenic, likely pathogenic, or a variant of

unknown significance by ACMG criteria, in the absence of another suspected genetic culprit (Tables II and IV in the Supplement). Fifty-one of 112 (45.5%) study participants were male and 91 (81.2%) were of European ancestry (Table 1). Among the 36 identified *CASQ2*-CPVT families, 9 had been previously reported. Details regarding all probands and family members provided in the IMCC cohort are provided in Table 2 in the Supplement.

In total, 12 probands were heterozygous for a *CASQ2* variant. Cascade screening was performed in 14 individuals from 6 of the 12 families and segregation of the variant with a CPVT phenotype was seen in 9 family members from 4 of the 6 families (Table II in the Supplement; Families 2, 5, 21, and 34). There were also 24 probands homozygous or compound heterozygous for *CASQ2* variants. For this group, clinical evaluation was performed in 37 of 52 heterozygous *CASQ2* family members and 8 had a positive CPVT phenotype (Table 1 and Table II in the Supplement; Families 10, 11, 14, 17, and 25). Overall, 51 of 66 *CASQ2* heterozygous family members underwent clinical evaluation with treadmill testing, epinephrine infusion, or Holter monitoring and 17/51 (33.3%) met CPVT diagnostic criteria. Heterozygous family members from families of a heterozygous proband were more likely to have adrenergic-induced ventricular arrhythmias relative to heterozygous family members of a homozygous or compound heterozygous proband (64.3% versus 21.6%, $p=0.007$; Table 1).

Clinical details for select probands and family members are provided in the following section.

***CASQ2* heterozygous probands and families**—The 12 *CASQ2* heterozygous probands can be grouped based on arrhythmic manifestations: 3 suffered an ACA (Table II in the Supplement; Families 21, 28, and 36), 3 experienced exercise-induced syncope (Table II in the Supplement; Families 2, 8, and 35), and 6 had pre-syncope, palpitations or chest pain and had exercise-induced ventricular ectopy consistent with CPVT (Table II in the Supplement; Families 5, 12, 19, 27, 32, and 34).

Among the 3 probands with ACA, the proband in Family 28 (*CASQ2*-p.R251H) presented with an ACA in association with exertion at 8 years of age and treadmill testing revealed polymorphic ventricular tachycardia (VT; Figure 1a). Following implantation of an ICD, the patient had recurrent shocks for exertion-induced polymorphic VT until initiation of a beta-blocker. Family 21 has previously been reported as an autosomal dominant form of CPVT secondary to a novel *CASQ2*-p.K180R variant identified with exome sequencing.²² A 4-year old male from Family 36 (*CASQ2*-p.W361R) was successfully resuscitated following a cardiac arrest that occurred during exertion and was subsequently found to develop frequent premature ventricular contractions and polymorphic VT on treadmill testing (Table II in the Supplement).

Among the 3 probands with adrenergic-induced syncope, genotype-phenotype segregation was observed in Family 2 (Table II in the Supplement; Family 2, *CASQ2*-p.R33*), which has been partially described in a previous report.²⁰ Of the 5 family members heterozygous for this variant, 2 exhibited exercise-induced ventricular ectopy consistent with a CPVT diagnosis.

For the remaining 6 CPVT probands, the proband of Family 5 (Figure 1b; CASQ2-p.E39*) presented with pre-syncope at 43 years of age and had polymorphic VT on treadmill testing (Figure 1c), while his 42 year old brother and 68 year old mother with the same variant developed ventricular ectopy during treadmill testing (Figures 1d and e). All 3 individuals heterozygous for the CASQ2-p.D340* variant from Family 34 (Figure 1f) had exertion-induced ventricular bigeminy (Figures 1g, h, and i). These investigations were triggered after the 9 year old female proband with exercise-induced palpitations had non-sustained polymorphic VT on Holter monitoring. Cascade screening in the remaining 4 families was either not possible or declined, including for the CASQ2-p.D325E proband with non-sustained polymorphic VT on treadmill testing (Figure 1j)

CASQ2 Homozygous and Compound Heterozygous Probands and Families—

Among 34 individuals (24 probands and 10 family members) homozygous or compound heterozygous for a pathogenic/likely pathogenic *CASQ2* variant or *CASQ2* variant of unknown significance, penetrance for a CPVT phenotype was 97.1% (33/34). The only patient without a positive clinical phenotype was a 4 year old female who had a normal Holter monitor, but had yet to undergo treadmill testing (Family 14; S113Rfs*6/S113Rfs*6). Twenty-six of the 34 (76.5%) homozygotes and compound heterozygotes had syncope, ACA, and/or SCD, with a median age of onset of 7 years (95% CI: 6–11).

Of these 24 families, 5 included at least one heterozygous family member (8 total) with a positive CPVT phenotype (Table II in the Supplement; Families 10, 11, 14, 17, and 25), however only 2 of the 8 had symptoms (details provided in the Online Supplement).

Impact of CASQ2 Genotype on Phenotype—Relative to *CASQ2* heterozygotes, *CASQ2* homozygote and compound heterozygote genotype status was associated with a 15.0-fold (95% CI: 5.7–39.4.3, $p < 0.001$) increased hazard of experiencing the composite outcome with syncope, though the results differed markedly when stratified by familial status (probands and family members). The increased hazard was 3.2-fold (95% CI: 1.3–8.0, $p = 0.013$) among probands alone (Figure 2A), but 38.8-fold (95% CI: 5.6–269.1, $p < 0.001$) in genotype positive family members (Figure 2B). Notably, all first presumed primary arrhythmic events in probands and genotype positive family members occurred prior to both initiation of β -blockade and insertion of an ICD.

For the composite arrhythmic outcome without syncope, *CASQ2* homozygote/compound heterozygote genotype status was associated with a 4.1-fold increased hazard of the outcome (95% CI: 1.0–16.2, $p = 0.045$) relative to heterozygotes. Stratification of the analyses by familial status showed no difference when the analysis was restricted to probands (HR: 0.9, 95% CI: 0.2–3.5, $p = 0.856$) (Figure 2C), whereas homozygous and compound heterozygous family members had an increased, albeit non-significant, risk relative to heterozygotes (HR: 8.7, 95% CI: 0.7–112.3, $p = 0.097$; Figure 2D).

Systematic Literature Review of Reported Cases

Literature review identified 2 additional families that included *CASQ2* heterozygotes manifesting cardiac syncope. Of the 2 reported variants - CASQ2-p.K206N and a large genomic deletion [1:116,242,628–116,311,402] involving exons 9–11 of *CASQ2* -

arrhythmia consistent with a positive CPVT phenotype was only reported in the p.K206N family.^{21,35} Full details are provided in Table III in the Supplement.

In Silico and Biochemical Evaluation of CASQ2 Variants

Population allele frequencies imply a low penetrance for heterozygous CASQ2 variants—From a combination of the IMCC and literature review, 55 presumed culprit *CASQ2* variants were identified, including 22 missense, 32 truncating (10 nonsense, 13 splice site/region, and 9 frameshift), and 1 large deletion (Table IV in the Supplement). Among the 55 variants identified, 29 had been previously reported (Table IV in the Supplement). The allele frequencies of the variants in gnomAD ranged from 0 (novel) to 0.06424% (p.D310N). The collective frequency in gnomAD of all presumed culprit *CASQ2* variants from the IMCC and literature review was 0.0997%, while that of all truncating *CASQ2* variants in gnomAD is 0.049%. In contrast, given an estimated CPVT prevalence of 0.01% and the consensus that *CASQ2* accounts for up to 5% of CPVT, *CASQ2*-CPVT should have a maximal prevalence of 0.0005%. Thus the collective prevalence of presumed pathogenic *CASQ2* variants in gnomAD is at least 398-fold greater than the expected prevalence of *CASQ2*-CPVT. Details of *in silico* prediction of variant deleteriousness and ACMG classification are provided in the Online Supplement.

Biochemical and Structural Analyses—Among the 16 missense variants identified in our novel case series, 7 manifested a positive CPVT phenotype in a heterozygous state: CASQ2-p.Y55C, -p.R251H, -p.P308L, -p.D325E, -p.W361R, and the previously reported CASQ2-p.S173I and -p.K180R variants.^{13,22} We performed both turbidity assays and SEC in order to characterize filamentation defects for 6 of the 7 putative dominant missense variants (cells transformed with the CASQ2-p.W361R clone failed to express the recombinant protein efficiently in culture) and compared findings with the recessive CASQ2-p.R33Q variant known to have a dimerization defect.

Turbidity Assays Reveal Filamentation Defects for Candidate Dominant-Acting Variants—Turbidity assays performed under standard physiologic conditions, including a pH of 7.4, revealed that the recessive CASQ2-p.R33Q variant and 5 of the 6 candidate dominant missense variants exhibited filamentation defects, the exception being CASQ2-p.R251H (Figure 3A–H). Although initial conditions did not support a filamentation defect for p.R251H (Figure 3E), the known low pH near the sarcoplasmic reticulum luminal membrane and the fact that our cardiac calsequestrin filament was crystallized at low pH conditions, led us to hypothesize that filament formation at low pH may be physiologically relevant to CASQ2 function.¹³ When assayed at a lower pH (5.6), a kinetic defect for p.R251H became apparent (Figure 3F).

Sizes Exclusion Chromatography (SEC) Reveals Dimerization of Candidate Dominant Variants Remains Intact—Given that apparent dominant and recessive missense variants exhibit filamentation defects, we subsequently utilized SEC in an effort to distinguish pathogenic variants that impeded front-to-front dimerization, and thus would favor a monomer state, from those that dimerize in a normal fashion. The recessive CASQ2-p.R33Q and -p.D307H variants have previously been shown to be defective at dimerization

under varying calcium conditions.¹¹ The SEC elution profile of wild-type CASQ2 revealed 3 peaks that are hypothesized to represent, from right to left, monomer, dimer, and tetramer species (Figure 4A). CASQ2-p.R33Q revealed an extra species between the monomer and dimer peaks, which may be reflective of its filamentation defect being secondary to impaired front-to-front dimerization (Figure 4A). In contrast, the other variants lacked this intermediate peak. Further, 5 of the 6 putative dominant missense variants, the exception being CASQ2-p.Y55C, had elution profiles revealing dimer levels that were comparable relative to wild-type, (Figure 4B–H). These findings suggest that the filamentation defects observed for these 5 missense variants do not occur secondary to an inability to undergo front-to-front dimerization and likely belong to a different mechanistic class.

Structural Analysis Reveals Candidate Dominant Variants Localize to Regions Relevant for Filamentation—We next mapped all 22 *CASQ2* missense variants previously implicated in CPVT to the newly determined crystal structure of the cardiac calsequestrin filament.¹³

Of the 6 candidate dominant variants evaluated functionally, the p.S173I, p.K180R, and p.D325E variants all localized to a solvent cavity formed by the inter-dimer interface (Figure 5A). In contrast, the CASQ2-p.R251H and -p.P308L variants localized to other parts of the filament structure (Figure 5B–C) and their filamentation defects were hypothesized to occur secondary to interference with packing of the CASQ2 dimer, considered critical for filament formation. Prior studies have shown that the dimer can occupy two different conformational states. Specifically, a more tightly-packed conformation is produced by divalent cation binding in the acidic groove between dimers, resulting in an assembly that is narrower by several angstroms.¹³ Structural alignment of loosely-packed dimers would be incompatible with the filamentated state due to the widened dimension of the longest axis of the dimer (Figure 5D). Consistent with this hypothesis, the histidine substitution at R251 localizes to the solvent cavity between the dimers where it could alter the packing of the 2 chains. Similarly, a leucine substitution at P308 may alter the location of nearby acidic residues that promote close packing by coordinating the cations that line the electronegative cavity between the dimers. In our recent study, multivalent ions were shown to line a narrow groove between dimer chains and the essentiality of multivalent coordination by D310, among others, is demonstrated for the purpose of tight packing of the dimer, allowing otherwise strongly repulsive electronegative surfaces to come into close contact.¹³ Notably, a prior molecular dynamics simulation of the P308L variant found changes in the packing of the adjacent acidic residues caused by partial unfolding of the short helix at P308.³⁷

The locations and putative functional categories of the remaining *CASQ2* missense variants are provided in Figure 6. In total, 9 missense variants exclusively observed to be transmitted in an autosomal recessive fashion localized to either the intra-dimer interface or the hydrophobic core (Figure 6). The CASQ2-p.P308Q variant, anticipated to impair dimer packing, was observed in a single compound heterozygote in the IMCC cohort and hence potential dominant behaviour could not be assessed clinically. The functional impact of the remaining missense variants was considered unknown.

CASQ2-p.Y55C Shows Improved Dimerization in a Reduced Environment—The Y55C variant, whose functional category remained unclear after initial structural analysis, is located within a β -strand that runs close to the hydrophobic core of thioredoxin domain I and extends out to the solvent-exposed surface at Y55. We hypothesized that the filamentation defect associated with the CASQ2-p.Y55C variant may be secondary to an alternative mechanism involving oxidization of the mutant cysteine residue due to its partially exposed location (Figure 1A in the Supplement). This would result in monomer-to-monomer or dimer-to-dimer disulfide bridges and subsequent concatemer formation. In order to evaluate this possibility, we repeated the turbidity assay for Y55C in the presence of 1mM of TCEP, a reducing agent, which partially rescued the filamentation defect (Figure 1B in the Supplement). SEC performed for CASQ2-p.Y55C in the presence of TCEP also partially normalized findings relative to wild-type through an apparent reduction in higher molecular weight species, potentially secondary to a reduction in disulfide concatemer formation (Figure 1C in the Supplement).

Discussion

Our international multi-center evaluation of *CASQ2*-CPVT is the largest clinical investigation for this genetic subtype of the arrhythmia and redefines its heritability on the basis of clinical findings that are bolstered by functional and structural data. Although often less severe relative to homozygous and compound heterozygous *CASQ2*-CPVT, our findings strongly support the possibility of a serious arrhythmic phenotype in heterozygotes, underscoring a need for universal clinical evaluation of all individuals found to possess a pathogenic *CASQ2* variant. The mechanisms responsible for a positive CPVT phenotype in the presence of a heterozygous *CASQ2* variant are likely heterogeneous, but appear to be intrinsic to the physical location and function of certain residues within the *CASQ2* filament. Other *CASQ2* variants appear to most often predispose to recessive inheritance patterns, though may yield a positive phenotype in certain heterozygous individuals, potentially secondary to a vulnerable genomic background or environmental influences.

Among *CASQ2* heterozygotes exhibiting a positive arrhythmic phenotype, the spectrum of severity was broad, ranging from SCD and ACA to incidentally detected ventricular ectopy on treadmill testing. Notably, the event rates observed for probands are not anticipated to reflect the true intrinsic arrhythmic risk imparted by most heterozygous variants in the general population due to selection bias.^{39,40} This concept is supported by the much lower event rates observed for *CASQ2* heterozygous family members and is further reinforced by the collective frequency of all presumed pathogenic *CASQ2* variants from the current report and the prior literature in gnomAD being 0.0997%, which is at minimum nearly 400-fold greater than the anticipated prevalence of *CASQ2*-CPVT. The apparent low penetrance alludes to a probable need for additional genetic and/or environmental factors in order for the majority of heterozygous *CASQ2* variants to manifest with a malignant phenotype, though there appear to be important exceptions.

Although truncating *CASQ2* variants anticipated to result in haploinsufficiency likely require additional genetic and/or environmental factors to manifest with an arrhythmic phenotype given their collective frequency in gnomAD of 0.049%, certain *CASQ2* missense

variants may be sufficient in isolation to cause an autosomal dominant form of CPVT through a dominant negative mechanism. This concept was originally suggested in a report involving the CASQ2-p.K206N missense variant and was recently bolstered by the dramatic autosomal dominant pedigree reported for the CASQ2-p.K180R variant.^{21,22}

The hypothesis that monomers that fail to undergo front-to-front dimerization are trafficked out of the sarcoplasmic reticulum and degraded, effectively rescuing the phenotype, alludes to the possibility that variants impairing back-to-back polymerization, but not front-to-front dimerization, may lead to a dominant negative effect.^{23–25} In our current report, we functionally evaluated 6 putative dominant missense variants found to impart filamentation defects, and SEC suggested that all, with the possible exception of CASQ2-p.Y55C, could effectively dimerize. Notably, the CASQ2-p.K180R, -p.S173I, and -p.D325E variants all localize to an electronegative solvent cavity responsible for back-to-back binding of dimers necessary for polymerization, a realization that was facilitated following recent revisions to the crystal structure of the cardiac calsequestrin filament.¹³

The remote locations of other candidate dominant missense variants from the inter-dimer interface indicates that there are likely heterogeneous mechanisms accounting for a dominant mode of inheritance. Similar to the presumed explanation for null alleles, it is conceivable that genomic background plays a critical role for some of these missense variants, particularly given that many family members were phenotype negative; however additional mechanisms may also be operative. For the CASQ2-p.R251H and -p.P308L variants, structural analysis suggested that they may interfere with packing of the dimer, leading to a looser conformation incapable of undergoing robust filamentation. The putative dominant behaviour of the CASQ2-p.Y55C variant may occur secondary to oxidation of the partially exposed mutant cysteine residue leading to the formation of inappropriate disulfide bridges and large concatemers. These dysfunctional concatemers may be retained within the sarcoplasmic reticulum and subsequently disrupt normal calcium-induced CASQ2 filamentation. The functional consequence of the CASQ2-p.W361R variant remains unclear, whereas the pathogenic effect of the previously reported CASQ2-p.K206N variant has been suggested to be mediated through a unique glycosylation-based mechanism.²¹

Based on our collective study findings, we support clinical evaluation of all individuals identified to be heterozygous for a pathogenic *CASQ2* variant. However in contrast to the guideline recommendation that presence of a pathogenic *RyR2* variant in isolation is sufficient for a diagnosis of CPVT, we would advocate that a diagnosis of CPVT only be assigned to an individual heterozygous for a pathogenic *CASQ2* variant in the presence of a positive clinical phenotype.²⁷ Although our findings confirm that heterozygous *CASQ2* variants may be arrhythmogenic, 34 of 51 family members had normal clinical testing indicating that the clinical penetrance of the majority of heterozygous *CASQ2* variants is low. Coupled with their benign clinical outcomes, our findings suggest that phenotype negative individuals likely do not require medical therapy or exercise restriction, though intermittent clinical screening to ensure they remain phenotype negative may be reasonable.

Limitations

Although the current study provides compelling data that heterozygous *CASQ2* variants may manifest with an arrhythmic phenotype, penetrance for the majority of variants appears highly variable suggesting that additional genetic and environmental factors may be operative. Future studies will be necessary to attempt to identify potential modifiers of an arrhythmic phenotype. It should also be noted that, although the expert consensus statement²⁷ indicates that first-degree relatives of a CPVT proband with exercise-induced premature ventricular contractions should be diagnosed with CPVT, this finding could be non-specific, particularly among older individuals with structural heart disease. Although this could result in penetrance being overestimated, we believe this to be unlikely in the IMCC cohort given the young age of patients, coupled with cardiomyopathy and coronary artery disease being exclusion criteria.

The findings from our biochemical and structural evaluations suggest that dominant inheritance patterns may occur secondary to a series of putative and heterogeneous mechanisms that impair *CASQ2* filamentation. These conclusions, however, are supported principally by *in vitro* biochemistry (turbidity assays demonstrating filamentation defects for the disease-associated variants). Studies of *CASQ2* variants in eukaryotic model systems are needed to confirm hypothesized electrophysiologic defects. Studies in cellular models may additionally identify cases of haploinsufficiency that occur due to degradation of poorly-folded mutant proteins, which would be distinct from our postulated mechanisms of interference with multimer formation.

Conclusions

This international multi-center study evaluating inheritance patterns in *CASQ2*-CPVT has revealed that heterozygous *CASQ2* variants may manifest with a malignant arrhythmic phenotype, indicating that *CASQ2* heterozygotes should undergo clinical evaluation in order to assess arrhythmic risk. Although certain heterozygous *CASQ2* missense variants may be sufficient to lead to an arrhythmic phenotype in isolation, the overall penetrance for most pathogenic heterozygous variants appears low, which suggests that patient management should be primarily guided by phenotype, rather than genotype alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

CPVT	catecholaminergic polymorphic ventricular tachycardia
SCD	sudden cardiac death
IMCC	International Multi-Center CASQ2
ACMG	American College of Medical Genetics and Genomics
gnomAD	Genome Aggregation Database
SEC	size exclusion chromatography
ICD	implantable cardioverter-defibrillator
ACA	aborted cardiac arrest

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Clinical Perspective

What is new?

- Pathogenic heterozygous *CASQ2* variants may manifest with a CPVT phenotype, indicating a need to clinically screen these individuals, though penetrance is variable.
- A dominant mode of inheritance appears intrinsic to certain missense variants owing to their location and function within the *CASQ2* filament structure.

What are the clinical implications?

- All individuals possessing a pathogenic *CASQ2* rare variant should undergo clinical evaluation to screen for a CPVT phenotype
- Among individuals heterozygous for a pathogenic *CASQ2* rare variant, medical therapy and exercise restriction is likely not necessary in the absence of a CPVT phenotype, though intermittent clinical screening to ensure they remain phenotype negative may be reasonable

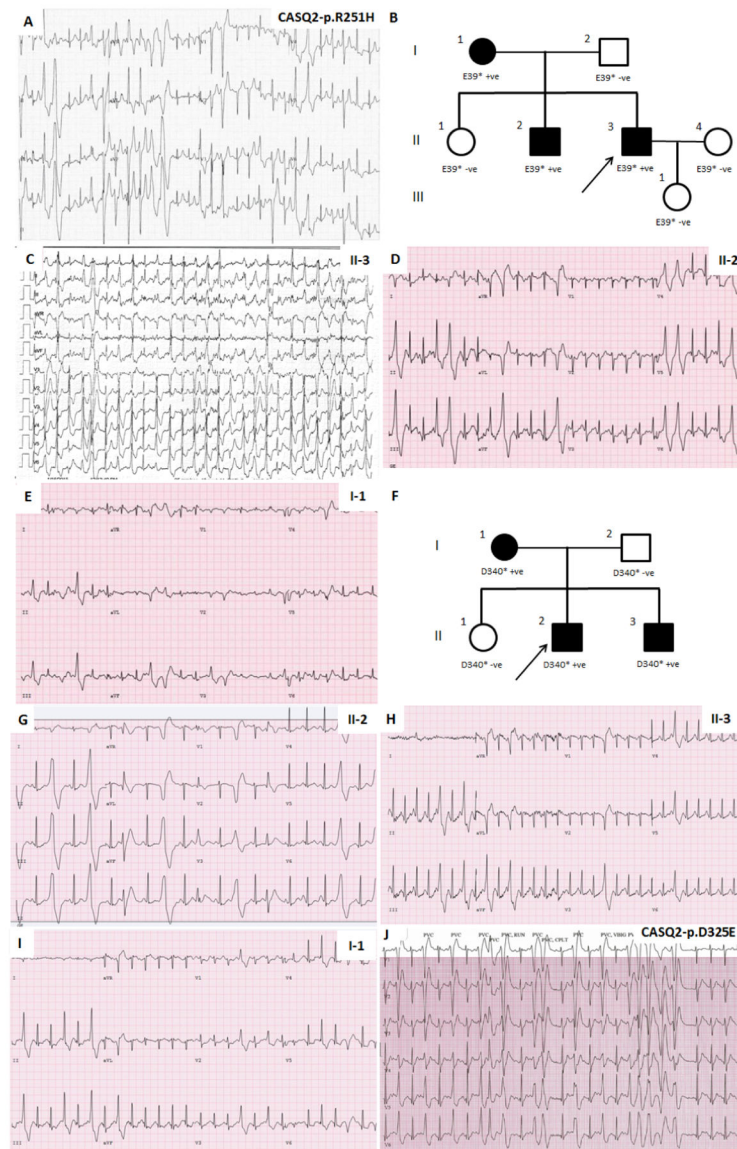


Figure 1: Clinical Phenotypes of *CASQ2* heterozygotes. **A)** Treadmill test of *CASQ2*-p.R251H heterozygous proband revealing non-sustained polymorphic ventricular tachycardia during exertion. **B)** Pedigree of the *CASQ2*-p.E39* family, **C)** Treadmill tests during the exertion phase of the *CASQ2*-p.E39* proband revealing non-sustained polymorphic ventricular tachycardia and evidence of prominent ventricular ectopy observed in his 42 year old brother (**D**) and 68 year old mother (**E**). **F)** Pedigree of the *CASQ2*-p.E39* family, Treadmill tests during the exertion phase of *CASQ2*-p.E39* proband (**G**) and family members revealing prominent ventricular ectopy (**H, I**). **J)** Treadmill test of the *CASQ2*-p.D325E proband revealing non-sustained polymorphic ventricular tachycardia during exertion.

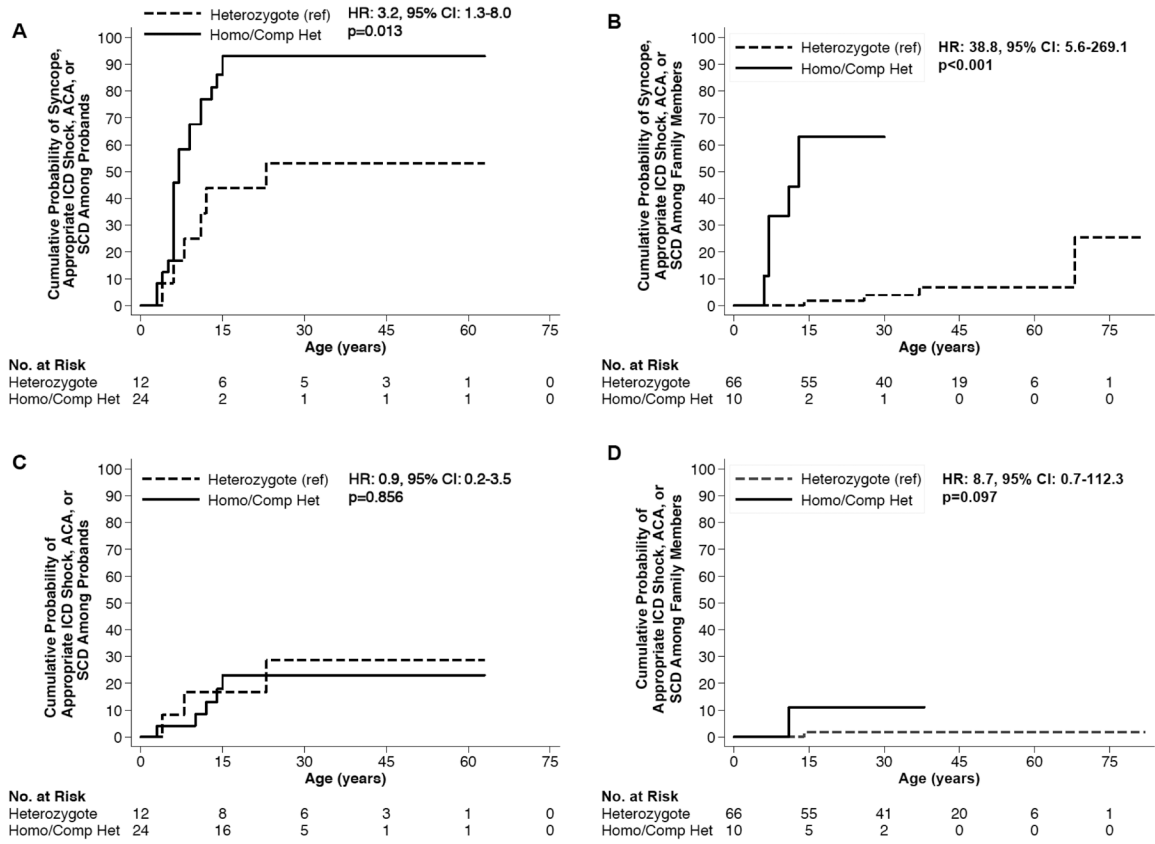


Figure 2: Arrhythmic Events Among Probands and Genotype Positive Family Members Possessing Putative Pathogenic Homozygous, Compound Heterozygous, or Heterozygous *CASQ2* Variants. Outcome of Syncope, Appropriate ICD Shock, ACA, or SCD Among Probands (A) and Genotype Positive Family Members (B) and Outcome of ICD Shock, ACA, or SCD Among Probands (C) and Genotype Positive Family Members (D) Homo/Comp Het = Homozygous/Compound Heterozygous, ICD = implantable cardioverter-defibrillator, ACA = aborted cardiac arrest, SCD = sudden cardiac death, ref = reference, HR = hazard ratio, CI = confidence intervals.

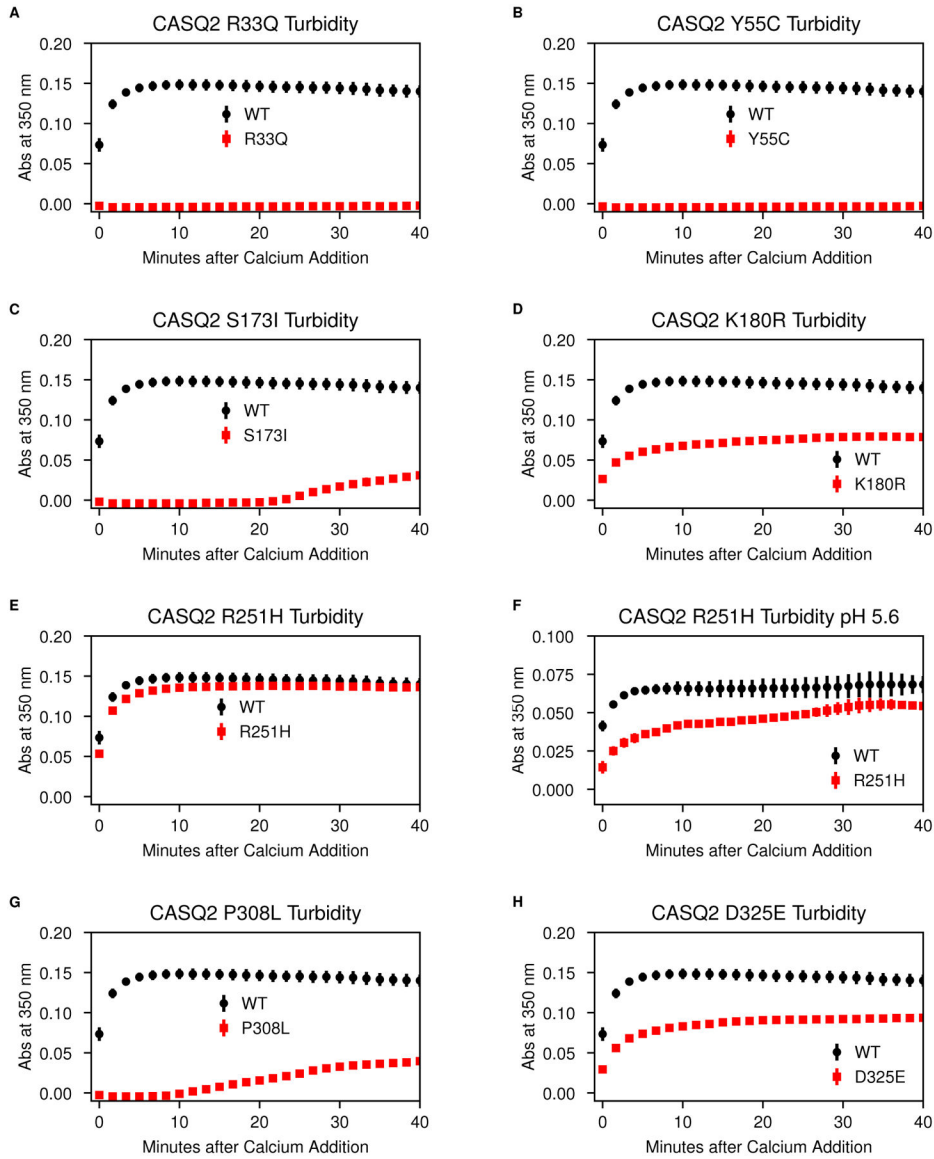


Figure 3: Dominant-acting CPVT-associated CASQ2 variants are associated with impaired filamentation as measured using a turbidity assay under physiologic-like ionic conditions. The previously reported recessive CASQ2-p.R33Q variant, known to impair filamentation by interfering with dimerization, is assayed as a control (A). Putative Dominant-Acting CASQ2 variants: Y55C (B), S173I (C), K180R (D), R251H (E), R251H assayed at pH 5.6 (F), P308L (G), D325E (H). Turbidity assays performed at 85mM KCl, 2mM MgCl₂, and 1mM CaCl₂. CPVT = catecholaminergic polymorphic ventricular tachycardia

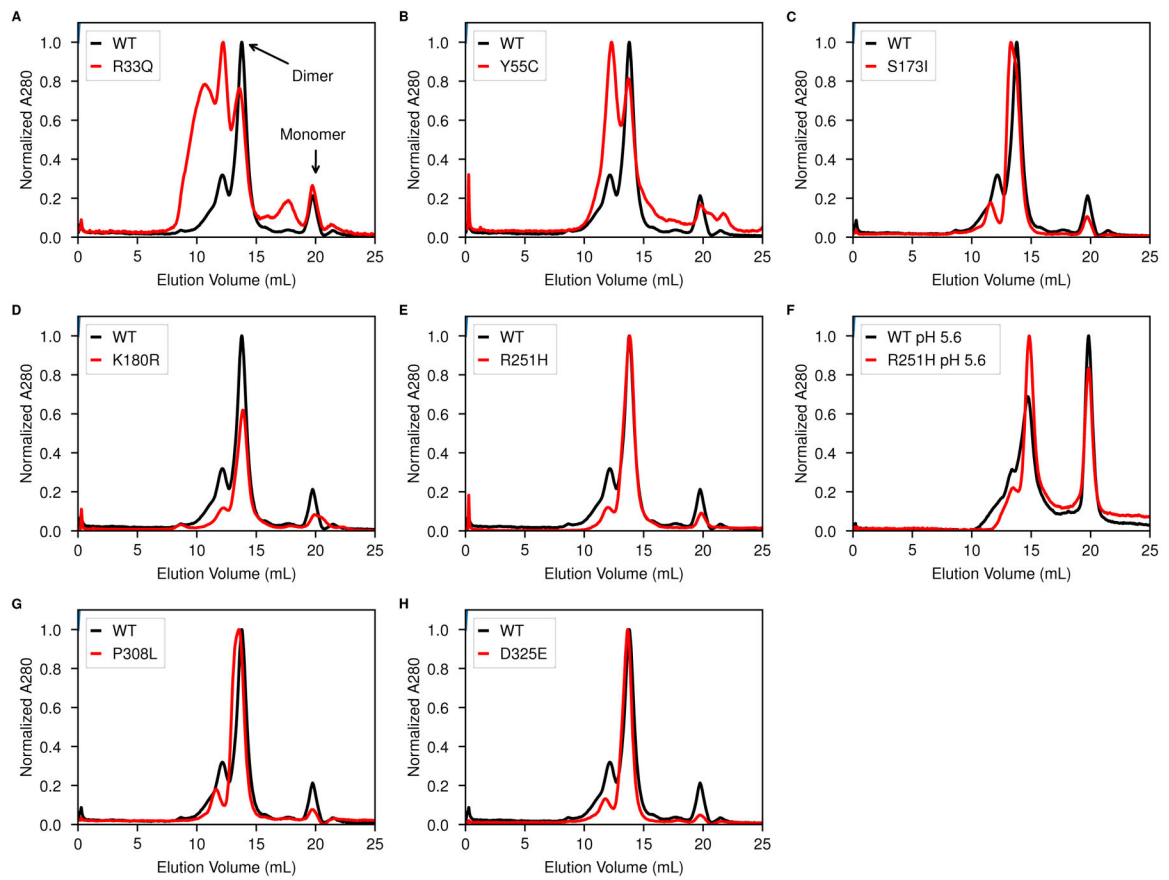


Figure 4:

Size-exclusion chromatography (SEC) provides evidence that putative dominant-acting CASQ2 CPVT variants have similar dimer/tetramer equilibria in the non-filamented state, with no evidence for interference with dimerization, in contrast to the recessive CASQ2-p.R33Q variant. R33Q (A), Y55C (B), S173I (C), K180R (D), R251H (E), R251H assayed at pH 5.6 (F), P308L (G), D325E (H). SEC at 85mM KCl and 1mM EDTA. CPVT = catecholaminergic polymorphic ventricular tachycardia

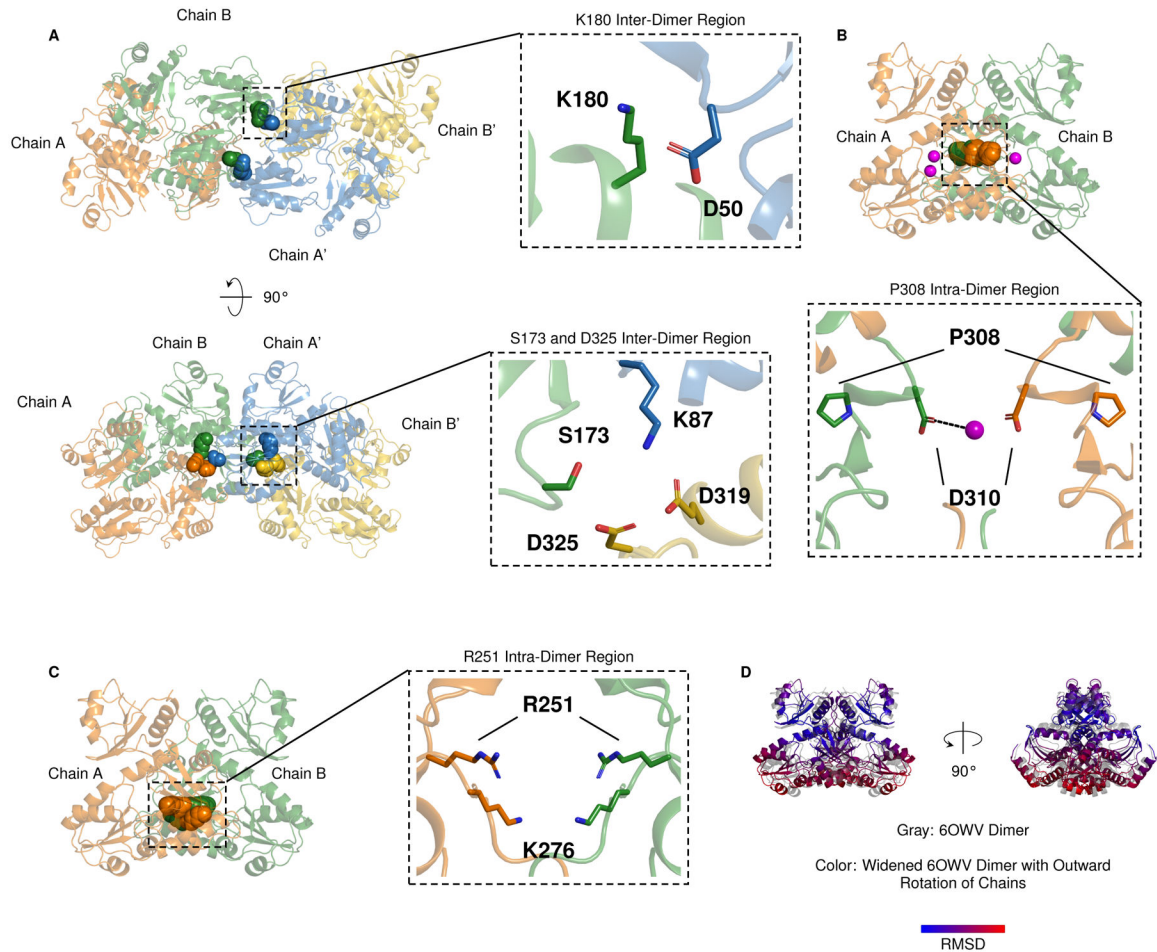


Figure 5: Visualization of known and putative dominant-acting CPVT-associated *CASQ2* variants within the *CASQ2* protein filament structure. (A) Dominant-acting CPVT-associated *CASQ2* variants p.S173I, p.K180R, and p.D325E disrupt the inter-dimer interface of *CASQ2* filaments. (B) and (C) Other putative dominant-acting variants (R251H, P308L) are hypothesized to act via a different mechanism. (D) Overlay of a loosely-packed *CASQ2* dimer (color) onto a tightly-packed dimer (gray), showing that the loosely-packed dimer is expanded, with its larger dimension possibly incompatible with robust filamentation. The cardiac calsequestrin structure from Protein Data Bank ID: 1SJI was used as a loosely-packed prototype for structural alignment. Magenta spheres indicate likely calcium positions identified by lanthanide substitution in Protein Data Bank structure 6OWV. 6OWV and 6OWW = Protein Data Bank IDs assigned to our newly reported human cardiac calsequestrin crystal structures¹³, 1SJI = Protein Data Bank ID of a previously reported cardiac calsequestrin structure³⁶, RMSD = root-mean-square deviation of atomic positions.

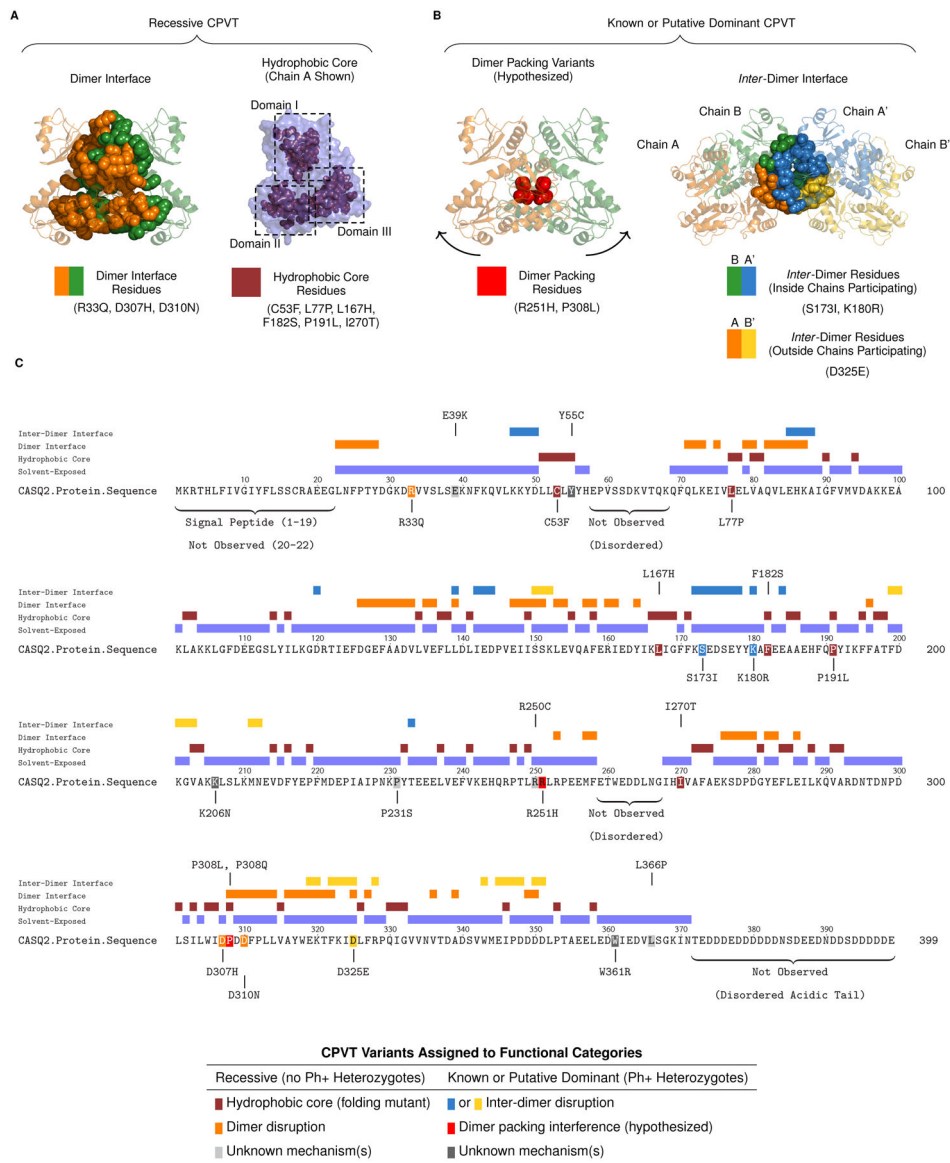


Figure 6: Locations of CPVT-Associated Missense Variants within the Cardiac Calsequestrin Protein and its Associated Domains. **A)** Rendering of protein domains associated with recessive CPVT variants. These include the intra-dimer interface and the hydrophobic core. **B)** Rendering of protein domains associated with known and putative dominant CPVT variants. These include residues hypothesized to contribute to dimer packing, as well as residues at the inter-dimer interface. **C)** CASQ2 protein sequence with delineation of amino acids present within the aforementioned domains (hydrophobic core, intra- and inter-dimer interfaces). Domains are indicated by colors corresponding to panels A and B. For interfaces that are symmetry related, only one set of colors is used. Pathogenic CASQ2 mutations implicated in CPVT are highlighted and categorized by putative functional effect. Buried surface area, as calculated by PISA, was used to assign residues to interfaces. Residues with < 4 square angstroms of solvent-exposed surface area in the CASQ2 monomer were

assigned to the hydrophobic core. CPVT = catecholaminergic polymorphic ventricular tachycardia, Not Observed (Disordered) = region not observed due to disorder in the crystal structure, Ph+ = phenotype positive. PISA = Proteins, Interfaces, Structures and Assemblies program.³⁸

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Table 1:

Clinical Features of Probands and Genotype Positive Family Members from Homozygous/Compound Heterozygous and Heterozygous Families with *CASQ2*-Catecholaminergic Polymorphic Ventricular Tachycardia

Clinical Variable	CASQ2 Homo/Comp Het Families			CASQ2 Het Families	
	Probands n = 24	Homo/Comp Het FM n = 10	Het FM n = 52	Probands n = 12	Het FM n = 14
Male (%)	14 (58.3)	4 (66.7)	20 (62.5)	7 (58.3)	6 (42.9)
European Ancestry (%)	19 (79.2)	7 (70)	41 (78.8)	11 (91.7)	13 (92.9)
Treadmill Test, Epi Infusion, or Holter Performed	24 (100)	9 (90)	37 (71.2)	12 (100)	14 (100)
Adrenergic-Induced PVCs	24 (100)	8/9 (88.9)	8/37 (21.6)	12 (100)	9 (64.3)
Adrenergic-Induced V Big	22 (91.7)	8/9 (88.9)	3/37 (8.1)	12 (100)	7 (50)
Adrenergic-Induced PVT or BiD VT	22 (91.7)	5/9 (55.6)	2/37 (5.4)	12 (100)	2 (14.3)
Cardiac Event	21 (87.5)	5 (50)	2 (3.8)	6 (50)	2 (14.3)
Age at first event (years)	7.9 ± 3.3	8.8 ± 3.0	52.5 ± 21.9	10.7 ± 6.7	20 ± 8.5
Cardiac Syncope	21 (87.5)	5 (50)	2 (3.8)	6 (50)	2 (14.3)
Aborted Cardiac Arrest	5 (20.8)	0 (0)	0 (0)	3 (25)	1 (7.1)
Sudden Cardiac Death	0 (0)	1 (10)	0 (0)	0 (0)	0 (0)

Data are n (%) or mean ± SD. Homo = homozygous, Comp Het = compound heterozygous, Het = heterozygous, FM = family member, Epi = epinephrine, PVC = premature ventricular contraction, V Big = ventricular bigeminy, PVT = polymorphic ventricular tachycardia, BiD VT = bidirectional ventricular tachycardia