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Meta-analysis of cardiomyopathy-associated variants in troponin genes identifies loci and intragenic hotspots that are associated with worse clinical outcomes

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Abstract

Introduction: Troponin (*TNN*)-encoded cardiac troponins (Tn) are critical for sensing calcium and triggering myofilament contraction. *TNN* variants are associated with development of

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CONFLICTS OF INTEREST:

No conflicts of interest to declare.

LIMITATIONS

Studies compiled were variable in data collection and were performed retrospectively. Comprehensive clinical data were not always available for probands and patients may have been lost to follow-up. Given that *TNN* variants are rare, we were likely underpowered to detect the prognostic impact of *TNNI* variants. Further, when calculating variant frequencies based on cardiomyopathy subtype, it is possible that minor cardiomyopathy variant frequencies are inflated due to limited patients screened in the literature for these genes. Further, homozygous, compound, and double heterozygous cases were small in number which limits our statistical analysis. Despite these limitations, this study represents the largest and most comprehensive study to date.

cardiomyopathy; however, recent advances in genetic analysis have identified rare population variants. It is unclear how certain variants are associated with disease while others are tolerated.

Objective: To compare probands with *TNNT2*, *TNNI3*, and *TNNC1* variants and utilize high-resolution variant comparison mapping of pathologic and rare population variants to identify loci associated with disease pathogenesis.

Methods: Cardiomyopathy-associated *TNN* variants were identified in the literature and topology mapping conducted. Clinical features were compiled and compared. Rare population variants were obtained from the gnomAD database. Signal-to-noise (S:N) normalized pathologic variant frequency against population variant frequency. Abstract review of clinical phenotypes was applied to “significant” hotspots.

Results: Probands were compiled ($N=70$ studies, 224 probands) as were rare variants ($N=125748$ exomes; 15708 genomes, $MAF < 0.001$). *TNNC1*-positive probands demonstrated the youngest age of presentation (20.0 years; $P=0.016$ vs *TNNT2*; $P=0.004$ vs *TNNI3*) and the highest death, transplant, or ventricular fibrillation events ($P=0.093$ vs *TNNT2*; $P=0.024$ vs *TNNI3*; Kaplan Meir: $P=0.025$). S:N analysis yielded hot-spots of diagnostic significance within the tropomyosin-binding domains, α -helix 1, and the N-Terminus in *TNNT2* with increased sudden cardiac death and ventricular fibrillation ($P=0.004$). The inhibitory region and C-Terminus in *TNNI3* exhibited increased restrictive cardiomyopathy ($P=0.008$). HCM and RCM models tended to show increase calcium sensitivity and DCM decreased sensitivity ($P < 0.001$). DCM and HCM studies typically showed no differences in Hill coefficient but were decreased in RCM models ($P < 0.001$). All CM models showed mostly no changes to F_{max} ($P=0.239$).

Conclusion: *TNNC1*-positive probands had younger ages of diagnosis and poorer clinical outcomes. Mapping of *TNN* variants identified locations in *TNNT2* and *TNNI3* associated with heightened pathogenicity, RCM diagnosis, and increased risk of sudden death.

Keywords

Cardiomyopathy; signal-to-noise analysis; troponin; *TNNT2*; *TNNI3*; *TNNC1*; Troponin T; Troponin I; Troponin C

INTRODUCTION

Cardiomyopathies are a heterogeneous group of primary diseases of the heart muscle, that can predispose to heart failure and cardiovascular death, with risk of sudden cardiac death (SCD) at first presentation [1]. Although many classifications exist, the American Heart Association classifies primary cardiomyopathies by genetic, mixed genetic, and non-genetic variants. Structural genetic primary cardiomyopathies include hypertrophic cardiomyopathy (HCM), left ventricular non-compaction cardiomyopathy (LVNC), and arrhythmogenic right ventricular cardiomyopathy (ARVC). Mixed structural cardiomyopathies, or those with both genetic and non-genetic etiologies, include dilated cardiomyopathy (DCM) and restrictive cardiomyopathy (RCM) [2].

The etiology of the majority cardiomyopathies, independent of the clinical presentation, is believed to be genetic. Genetic variants resulting in molecular defects in the components of

the contractile myofilaments represent a major cause of cardiomyopathies [3, 4]. The cardiac troponin molecular complex (Tn) is essential for the regulation of striated muscle contraction and is located along the sarcomere thin filament [5, 6]. Disruption of any of these processes likely leads to cardiac dysfunction and cardiomyopathy.

While variants in *TNN*-encoding genes are clear causes of cardiomyopathy, there is marked clinical heterogeneity. Genotype-phenotype correlations to explain this heterogeneity have been limited by small numbers of probands and a lack of comprehensive knowledge of population variants. Recent genetic studies have identified previously reported pathologic variants in large population studies as well as rare variants of unknown disease risk [7, 8].

It is unclear how certain *TNN* variants are associated with disease while others are physiologically tolerated. To address this, we set out to 1) create a compendium of cardiomyopathy (CM)-associated variants and population-associated variants, and 2) identify prognostic characteristics associated with genotype.

METHODS

Nomenclature

Nomenclature in this study is detailed in Supplemental Materials.

Compendium of cardiomyopathy-associated *TNN* variants

To compile pathologic *TNN* variants, PubMed and the Human Gene Mutation database were queried through June 2018 for studies identifying patients with cardiomyopathy and confirmed *TNN* variants [9]. Inclusion criteria for the studies included 1) study of individuals with primary cardiomyopathic disease, 2) comprehensive genetic analysis of the coding exons of *TNNT2*, *TNNI3*, or *TNNC1*, 3) availability of individual variants and their amino acid location. A variant from these studies was included in our analysis if it was 1) hosted by a proband, 2) described in the sentinel study reporting the proband, and 3) was denoted as pathogenic or likely pathogenic by ACMG criteria. A variant was excluded if 1) non-proband cases hosted the variant, 2) the variant was available and denoted as “benign” or “likely benign” by ClinVar or was denoted as a variant other than pathogenic or likely pathogenic by ACMG criteria, further described in our Supplemental Materials [10]. The latter exclusion criterion was applied to control for recent changes in the assessment of variant pathogenicity. Given the evolution of pathogenicity among some variants over time, as well as heterogeneity among individual studies included, we manually applied current ACMG criteria to re-assess the pathogenicity of all variants, independent of any previously assigned classifications [11]. Further inclusion criteria for S:N analysis considered whether the study was cohort-based. Variants were excluded from S:N analysis if they were sense, synonymous, intronic, UTR, or intergenic. Variants were abstracted and standardized based on consensus primary sequence *TNNT2* (NP_001001430), *TNNI3* (NP_000354), and *TNNC1* (NP_003271) (Ensembl, release 93). Studies testing functional consequences of variants using animal models and *in vitro* assays were also compiled in a separate database [12].

Compendium of rare population-based *TNN* variants

To compile rare population variants in Tn, variants from the Genome Aggregation Database (gnomAD) were compiled [13]. These were utilized as “control” or “reference” alleles. Given the observation of likely pathogenic variants found among gnomAD cases, we determined the maximum credible minor allele frequency (MAF) of gnomAD-identified pathogenic variants and set an inclusion threshold of MAF <0.001. This MAF threshold was set to include any pathologic variants observed in gnomAD and has been validated for both heritable arrhythmias and cardiomyopathies [14–16].

Amino acid-level signal-to-noise analysis and structure modeling

Variant mapping and amino acid-level signal-to-noise (S:N) analysis was done as previously described and is fully detailed in the Supplemental Materials [17]. Following exclusion of *TNN* homozygous and compound and double heterozygote probands, variants were mapped along the protein topology for each protein and signal-to-noise calculations involved normalization of variant frequency against gnomAD variant frequency at each amino acid position. For each *TNN* gene, variant hotspots considered significant by S:N analysis were determined (Supplemental Methods). Probands with variants within each hotspot were identified and grouped by hotspot locations. These hotspots were further grouped based on functional domains. Clinical features of probands were compared amongst hotspots at each functional domain. A Bonferroni corrected threshold was set to determine clinically significant hotspots with outcome relevance.

Comparison Statistics

Fully detailed in Supplemental Materials.

RESULTS

Creation of troponin variant database and meta-analysis

To identify all pathologic variants and rare population variants, we created a compendium of cardiomyopathy-associated variants and gnomAD-based population variants (Figure 1A). A comprehensive review of the literature identified 224 probands hosting variants in *TNN* from 70 studies. A total of 54 distinct variants were identified in *TNNT2*, 53 in *TNNI3*, and 16 in *TNNC1* (Supplemental Table 1). Individual proband data for *TNNT2*, *TNNI3*, and *TNNC1* are detailed in Supplemental Tables 2, 3, and 4, respectively. Three hundred and five rare population variants were collected from the gnomAD database v2.1.1 (N=125748 exomes; 15708 genomes, MAF <0.001).

Frequency of pathologic troponin variants is higher among individuals with cardiomyopathy than rare population variants

While variants in *TNN* are classically believed to be rare among individuals with CM, CM-associated pathologic frequency was significantly higher in all individual *TNN* genes compared to all gnomAD variants, with frequencies of 1.35% (1.12–1.63), 1.21% (0.99–1.48), and 0.41% (0.24–0.69) in *TNNT2*, *TNNI3*, and *TNNC1*, respectively, compared to population cohorts of 0.32% (0.30–0.34), 0.18% (0.17–0.20), and 0.06% (0.05–0.07).

Comparisons among all frequencies were significant ($P < 0.001$). Variant frequencies are summarized in Figure 1B. Variant frequencies in *TNNT2* were 0.83% (0.50–1.36) for DCM, 1.33% (1.07–1.67) for HCM, and 4.17% (0.74–20.24) for RCM. In *TNNI3*, variant frequencies were 0.57% (0.34–0.98) for DCM, 1.35% (1.07–1.71) for HCM, and 19.44% (9.75–35.03) for RCM. In *TNNC1*, variant frequencies were 0.61% (0.28–1.34) for DCM, 0.24% (0.10–0.57) for HCM, and 1.83% (0.62–5.24) for LVNC. Variant frequencies were consistently lower in DCM and were higher in rarer cardiomyopathy subtypes such as RCM and LVNC across all genes. S:N for each gene was 4.22, 6.72, and 6.83 for *TNNT2*, *TNNI3*, and *TNNC1*, respectively. These results indicate that, while rare, pathologic variants in *TNN* genes are more prevalent in clinical cases than rare population variants, and suggest these variants have meaningful diagnostic signal-to-noise relevance if there is a clinical diagnosis of CM.

TNNC1*-variant positive probands have a worse prognosis compared to *TNNT2* or *TNNI3

To determine if the *TNN* gene mutated was correlated with clinical outcomes, we compared severity of cardiomyopathy and mortality among the three genes, looking specifically at heterozygous probands. *TNNC1* probands were diagnosed within Almost 40% of the first decade of life (Figure 2A). *TNNC1* probands were diagnosed at an earlier age with a mean age of 20.00 ± 5.26 years, as compared to 33.70 ± 1.99 years in *TNNT2* and 39.30 ± 2.49 years in *TNNI3* ($P = 0.016$ and $P = 0.004$, respectively; Figure 2B). Fatal and potentially fatal events including death, transplant, and documented ventricular fibrillation (VF) affected 53.80% (29.14–76.79) of *TNNC1* probands as compared to 26.50% (18.80–36.04) of *TNNT2* probands and 22.40% (14.80–32.29) of *TNNI3* probands ($P = 0.093$ and $P = 0.024$, respectively). Survival was compared and is plotted in Figure 2C (global $P = 0.025$; *TNNC1* vs *TNNI3*: $P = 0.008$; *TNNC1* vs *TNNT2*: $P = 0.019$). *TNNC1*-positive probands were more likely to have LVNC, with 23.10% (8.18–50.25) having LVNC as compared to 1.10% (0.19–5.84) in *TNNI3* and 1.00% (0.18–5.45) in *TNNT2* probands ($P = 0.001$ and $P = 0.010$, respectively; Figure 2D). Conversely, 15.00% (9.18–23.70) of *TNNI3* probands had RCM, as compared to 2.00% (0.55–7.00) of *TNNT2* and 0.00% of *TNNC1* probands ($P = 0.003$ and $P = 0.001$, respectively). Combined *TNNT2*, *TNNI3*, and *TNNC1* proband clinical and demographic data are detailed in Supplemental Table 5. Data analysis prior to exclusion of compound and double heterozygote and homozygote probands are detailed in Supplemental Table 6. Taken together, *TNNC1*-positive probands demonstrated a more malignant phenotype compared to their *TNNT2* and *TNNI3* counterparts.

Higher troponin variant zygosity conveys an earlier age of diagnosis and a negative prognosis

Given recent evidence that variant burden has prognostic relevance in cardiomyopathic disease, we next explored the impact of variant zygosity and compound and double heterozygosity on prognosis.[18, 19] Overall, 91.97% (87.66–94.86) of all *TNN*-positive probands were heterozygous and 1.34% (0.46–3.86) were homozygous for pathologic variants across all *TNN* genes, 2.23% (0.96–5.12) carried compound heterozygous variants, and 4.46% (2.44–8.02) were double heterozygote. Initially, we grouped all probands with homozygous variation and compound and double heterozygosity and compared them to those with heterozygous variants. Of clinical variables explored, age of diagnosis was 16.0

years (SEM: 3.50) in our higher variant burden group compared to heterozygous probands who had a mean age of 35.1 years (SEM: 1.54; $P<0.001$). Other variables were similar in both groups ($P>0.05$).

Although proband numbers were small when looking at each group separately, we next sought to address differences in clinical variables based on type of variant burden. We found that the majority of homozygous probands were transplanted or died, although only 3 probands were available (Supplemental Table 7). Death or transplant is higher among homozygous probands than those with heterozygous variants where only 8.20% (5.09–12.85) were transplanted and 24.50% (19.00–30.96) were either transplanted or died. Compound heterozygous probands were found to be younger at time of diagnosis than their single variant counterparts as well (8.20 ± 2.58 years vs 35.1 ± 1.54 years; Figure 3A; Supplemental Table 8). Double heterozygote probands also had younger ages of presentation (18.40 ± 5.83 years vs 35.1 ± 1.54 years) compared to those with single variants (Figure 3B; Supplemental Table 9). Overall, homozygosity, double heterozygosity, and compound heterozygosity were rare yet correlated with worse outcomes among all *TNN* genes.

Amino acid-level signal-to-noise analysis identifies variant hotspots that drive prognostic associations

Given the recent observation that pathologic *TNN* variants have been observed within the gnomAD database, we sought to integrate rare population variants to determine areas that may drive diagnostic and prognostic influence of the *TNN*CM-associated variants. Amino acid-level S:N has recently been shown as a method to normalize pathologic variation against the rare population variation to identify pathologic hotspots [17]. In this way, we sought to identify areas of pathologic and prognostic relevance distinct from rare population variation. Based on pre-analysis significance of double and compound heterozygote and homozygote probands, only heterozygote probands were included in this analysis.

Hotspots of diagnostic relevance were isolated in *TNNT2* within the N-terminus, tropomyosin binding domains, and α -helix 1 (Figure 4). Probands hosting variants at these locations demonstrated significant differences in sudden cardiac death/ventricular fibrillation (SCD/VF; $P=0.004$) and diagnosis ($P<0.001$), with SCD/death/transplant ($P=0.053$) and age ($P=0.052$) trending towards significance. Hotspot 3 (aa85–95; S:N= 310) proved to be highly diagnostically relevant, with 26.3% SCD/VF and 31.6% SCD/VF/death, with all probands having HCM. Hotspots 2 (aa73–80; SN: 31) and 15 (aa277–281, SN: 61) exhibited significantly higher SCD/VF. Hotspot 11 (aa204–206; S:N: 61) exhibited 100% transplant and a young age of diagnosis at 8.3y, although only two probands are identified at this location. Hot-spot 12 (aa209–211, SN: 17) also had a higher amount of SCD/death/transplant. Five different hotspots of clinical significance were isolated within vital functional domains. S:N hotspots are listed in Supplemental Table 10 and hotspots grouped by functional domains are listed in Supplemental Table 11.

TNNI3 proband hotspots were similarly compared. Most hotspots and probands clustered within the inhibitory region, switch peptide region, and the C-terminus (Figure 5). Hotspots of diagnostic relevance had higher rates of heart failure ($P=0.030$), as well as less common cardiomyopathy subtypes such as RCM ($P=0.008$). Hotspots 9 (aa140–146; S:N= 58), 12

(aa161–167; S:N= 96), 13 (aa169–187; S:N=225), and 14 (aa195–210; S:N=80) had, respectively, 60.0%, 90.0%, 100.0%, and 50.0% of probands who have heart failure. Hotspots 9 and 13 both have an increased amount of RCM with 13.3% and 9.5%, respectively. S:N hotspots are listed in Supplemental Table 12 and hotspots grouped by functional domains are listed in Supplemental Table 13.

TNNC1 proband hotspots were compared similarly. However, no hotspots of diagnostic significance were identified (Supplemental Figure 1). This is likely due to the small number of probands and/or high pathogenicity of all hotspots. S:N hotspots are listed in Supplemental Table 14.

Analysis of inheritance and variant alterations

We sought to analyze the effect of inheritance and polarity and charge changes on phenotype. This is detailed in the Supplemental results.

Functional Consequences of TnT, TnI, and TnC Variants

Functional characteristics of pathologic variants were compiled from 96 different studies. Testing of biochemical/biophysical properties of thin filaments and the troponin macromolecular complex were variable amongst most studies, although common themes were seen. We compared results of common studies including calcium sensitivity, F_{max} , and Hill coefficient. Statistical differences between CM models were exhibited in pCa_{50} , determined by concentration of Ca^{2+} at which the force is half of the maximal value and a marker of calcium sensitivity, and in Hill coefficient, a measure of cooperative action/cooperativity. For pCa_{50} , 68.0% of HCM models (83/122 models) and 76.9% of RCM models (20/26 models) demonstrated an increased pCa_{50} , while 52.5% of DCM models (21/40 models) showed decreased pCa_{50} ($P<0.001$). Conversely, 51.9% of HCM models (28/54 models) and 92.9% of DCM models (26/28 models) showed no change in the Hill coefficient while 60.0% of RCM models (9/15 models) showed decreases in Hill coefficient ($P<0.001$). No difference was found in F_{max} , a measure of maximal force, among cardiomyopathy types. Although data was scarce, HCM models tended to show increased maximal velocity of shortening, where changes were exhibited, and DCM decreased maximal velocity of shortening, where changes were exhibited. HCM and RCM models tended to show increase calcium sensitivity and DCM decreased sensitivity. This is fully detailed in Supplemental Table 16. Taken together, these findings suggest variants associated with specific types of cardiomyopathy impart similar biophysical effects.

DISCUSSION

Variant Investigation in Cardiomyopathy

In light of the rapid progression of precision or personalized medicine, efforts to utilize an individual's specific genetic abnormality to predict outcomes and guide therapy are underway. Thus, understanding both the functional and prognostic implications of rare variation is critical to the development of personalized medicine.[20] Recent studies have highlighted that specific genetic variation that is associated with the development of cardiomyopathy, in particular HCM, may guide rational drug design and the development of

novel pharmacotherapies [21]. For instance, chronic administration of the small molecule MYK-461 has been shown to reduce signs of HCM and reduce profibrotic gene expression in mice with heterozygous variants in the myosin heavy chain [22]. Further, establishing the diagnostic and prognostic impact of known pathologic variation on sarcomeric genes has formed the foundation for potential gene therapies. For example, CRISPR-Cas9 targeting of a GAGT deletion in *MYBPC3* in human embryos and expression of functional cMYBPC in *MYBPC3* knockout mice with use of AAV therapy [23, 24]. Overall, our findings that specific genetic variants were associated with specific prognostic outcomes reinforces the role of genetic testing in personalized care of cardiomyopathies.

Genetic investigations have revealed a large number of variants in Tn subunits linked to CM. Prognostic utility of genetic variants and their impact on phenotype has been controversial. Early studies have found that myofilament-positive HCM patients had more severe cardiac dysfunction, higher likelihood of progressive heart failure, and higher rates of cardiovascular death [25, 26]. More recently, it has become clear in larger cohort studies that sarcomere positive cardiomyopathy is associated with a poorer prognosis compared to their sarcomere negative counterparts, with higher endpoints of cardiovascular death, progression to end-stage heart failure, and non-fatal stroke [27, 28]. At the individual gene level, some early correlations have been identified such as heightened pathogenicity and SCD in *TNNT2* at residue 92 and heightened variant rates in codon 145 in *TNNI3* causing RCM or HCM [26, 29]. However, these multigenerational family studies have been difficult to replicate in large population studies. Nonetheless, significantly more probands identified in the literature will aid in determining gene-level prognostic correlations. With the advancement of genetic testing and newly discovered variants, we set out to determine *TNN* gene-specific prognostic associations.

Our compiled data demonstrated that *TNNC1* probands had the youngest age of diagnosis and the highest rates of potentially fatal events. *TNNT2* and *TNNI3* probands had similar rates of death, SCD, and documented VF, although *TNNI3* probands had a propensity for RCM. Although not statistically significant, more than 40% of *TNNC1* probands lacked a family history as compared to lower proportions in *TNNT2* and *TNNI3*. *De novo* variants play an important role in severe, early-onset diseases and are significant in sporadic schizophrenia, neurodevelopmental disorders, and congenital heart disease [30–33]. The combination of an earlier disease onset and increased negative outcomes in *TNNC1* probands could explain the increased amount of *de novo* variants. The probable explanation is survival to transmission of the mutant allele was less likely given its lethality. This would further be supported by the scarcity of *TNNC1* probands identified in the literature (7.6% of all *TNN* probands identified). Further, although outside of our study's set time period, recently identified *TNNC1* probands have exhibited similar findings in our study, including young ages of diagnosis, death, and negative family histories [34, 35]. Comparison of survival between each of the *TNN* genes demonstrated a significantly worse prognosis in individuals hosting likely pathologic variants in *TNNC1* compared to *TNNT2* and *TNNI3*.

Variant Burden

Variant burden is an important factor that affects clinical variability in variant-positive cardiomyopathy probands. Compound heterozygosity, double heterozygosity, and homozygosity are linked with more severe phenotypes, both in humans and animal models. Although less common than single variants, compound heterozygotes make up around 5% of HCM probands [36]. Presence of multiple variants in HCM patients has been linked to earlier onset of disease, a more severe phenotype, and a higher prevalence of major cardiac events [36–39]. Similar findings of increased SCD and arrhythmic events were found in ARVC associated with compound heterozygosity [40, 41]. Homozygosity in the HCM cohort is also associated with rates of SCD, and more significant hypertrophy [42]. Our analysis earlier onset of disease, higher cardiac further amplifies these findings. Compound heterozygotes and double heterozygotes, regardless of variant class, were associated with a younger age of diagnosis while homozygosity was associated with a higher rate of transplant and death, where over 50% of homozygous probands were transplanted and all probands were transplanted or died.

Insights with Signal-to-Noise Analysis

Pathologic variants within the *TNN* genes are frequently linked to significant functional changes. Recently, studies have exhibited that some pathologic variants are found within the general population [7, 8, 43, 44]. Our primary goal was to address recent observations of pathologic variants in population-based data by S:N analysis.

In TnT, distinct locations with poor prognosis were found within the N-terminus, the tropomyosin binding domains, and α -helix 1. *TNNT2* variants in or near the N-tail T1 domain (aa79–170) can directly interact with overlapping tropomyosin (Tm) [45]. Variants between 92 and 110 were found to impair tropomyosin-dependent functions of TnT and alter secondary structure in the regions of Ca^{2+} -dependent interaction between TnI and TnC [45, 46]. Hotspot 3 (aa85–95), located in this area, exhibited the highest S:N ratio in our analysis and was a highly pathogenic phenotype. The C-terminal region of TnT (i.e. TnT2 digest fragment) is known to interact with other Tn subunits and also binds the tropomyosin molecule; the last 14 amino acid residues are critical for stability of the inactive state [47, 48]. Interestingly, variant hotspots were located within the C-terminus of TnT, one within the first α -helix and another towards the C-terminal end, within 14 amino acids of the terminal end. In TnI, several hotspots were located within the C-terminus. The mobile domain (aa168–210 or aa163–210 as suggested by Yamada et al.) is vital in the interaction with actin and N-TnC and has been shown to stabilize interactions between TnI and actin in the relaxed state of thin filament [49–51]. Even deletion of the last three residues in the C-terminus is associated with significant functional compromise [52].

The most recent findings regarding *TNN* variants are in the *TNNC1* gene. Prognosis among most probands was significantly poor and lack of comparatively pathogenic hotspots is likely secondary to this. Interdomain communication within TnC may mean that single variants could affect function of multiple domains [53, 54]. However, an area of significance where variants are detrimental to protein function include the N-helix, an important modulator of Ca^{2+} binding affinity and myofilament regulation [55–58].

Although hotspot domain location may explain pathogenicity, Tn domain function and variant alterations are still unclear. Residue changes have functional consequences inherent in their location, but variants can also affect covalent, post-translational modifications and Tn proteins may have non-canonical roles [59, 60]. Further, our functional studies exhibited some common biochemical property alterations common in each cardiomyopathy subtype. This included heightened calcium sensitivity in HCM and RCM compared to DCM, where a decrease was seen. Heightened calcium sensitivity is thought to contribute to hypercontractility and have been linked to development of arrhythmias [61, 62]. RCM also exhibited decreased cooperative action of myofilaments, a process thought to have a diminishing effect on force development [63]. Regardless, functional consequences remain highly variable amongst variants studied.

In conclusion, our data exhibited that *TNNC1*-positive probands carried the poorest prognosis. Mapping of *TNN* variants revealed distinct locations in *TNNT2* and *TNNI3* associated with high risk of variant pathogenicity, early presentation, RCM diagnosis, and increased risk of VF and SCD. Future investigations should focus on *TNNC1* variants as more probands are discovered. Furthermore, probands with *de novo* variants, homozygosity, and compound heterozygotes warrant further prospective investigations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

DCM	dilated cardiomyopathy
HCM	hypertrophic cardiomyopathy
LVNC	left ventricular non-compaction cardiomyopathy
RCM	restrictive cardiomyopathy
S:N	signal to noise
SCD	sudden cardiac death
Tn	cardiac troponin molecular complex
TnC	Troponin C
TnI	Troponin I

TNN	Troponin Gene
TnT	Troponin T
VF	ventricular fibrillation

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CLINICAL PERSPECTIVES

In light of population-based data showing variants, it has become unclear how certain variants are well tolerated and others pathogenic. S:N analysis is a viable tool to better analyze variant hotspots, while integrating rare population variants. S:N analysis can be used to identify pathologic hotspots in other genes that demonstrate rare variants found in ostensibly healthy individuals.

Highlights:

- It is unclear how certain variants are well tolerated and others pathogenic.
- *TNNC1*-positive probands carried the poorest prognosis.
- Mapping of *TNN* revealed locations in *TNNT2* and *TNNI3* associated with pathogenicity.
- *De novo* variants, homozygosity, and compound heterozygotes have poorer prognosis.

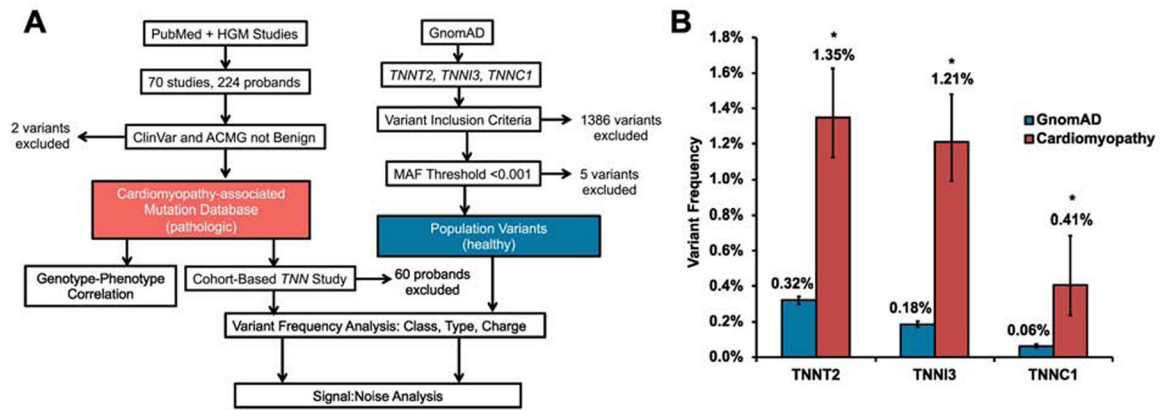


Figure 1: A. Study methodology and inclusion and exclusion criteria. **B.** Variant frequencies in variants and the gnomAD database. * $P=0.0001$

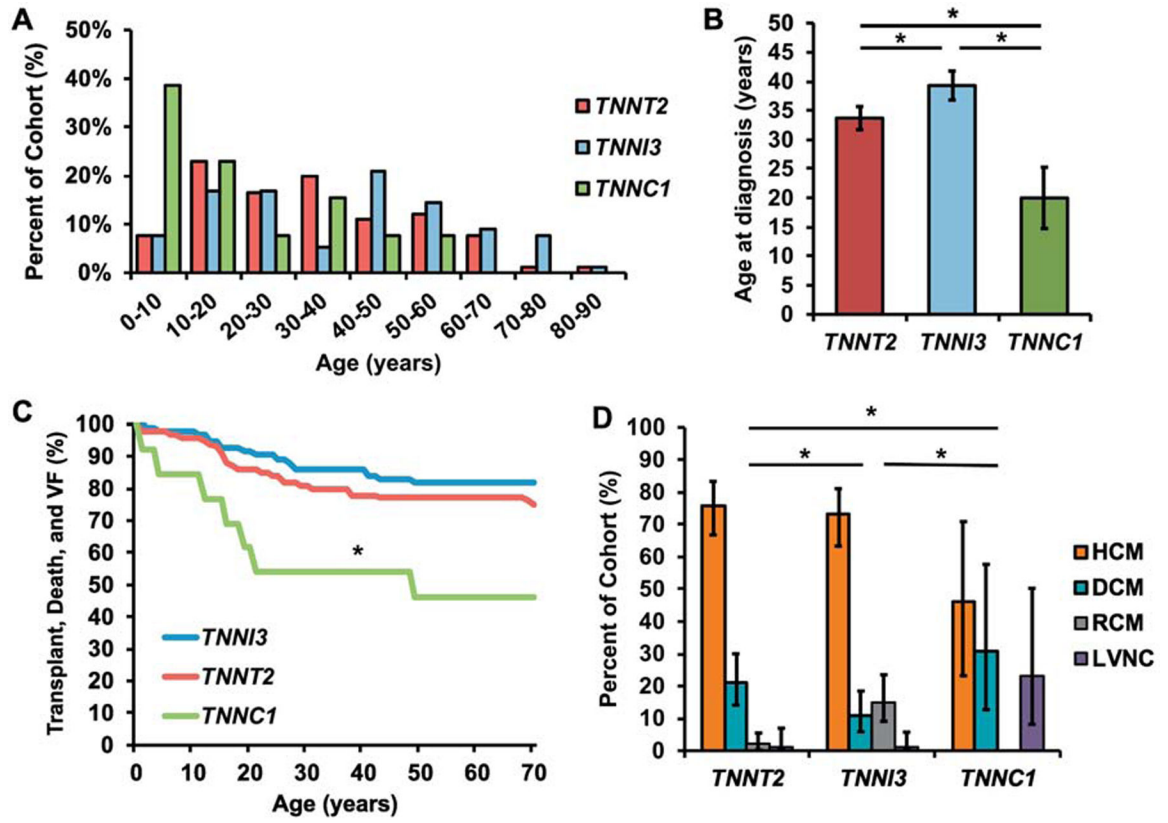


Figure 2: A. Histogram exhibiting distribution of ages of diagnoses in *TNNT2* (red), *TNNI3* (blue), and *TNNC1* (green) probands. **B.** Histogram exhibiting average proband age in *TNNT2*, *TNNI3*, and *TNNC1*. **C.** Survival curve for *TNNT2* (red), *TNNI3* (blue), and *TNNC1* (green) probands; * *TNNC1* vs *TNNI3*: $P=0.008$; *TNNC1* vs *TNNT2*: $P=0.019$. **D.** Histogram exhibiting the distribution of hypertrophic cardiomyopathy (HCM; orange), dilated cardiomyopathy (DCM; blue), restrictive cardiomyopathy (RCM; grey), and left ventricular noncompaction cardiomyopathy (LVNC; purple) in *TNNT2*, *TNNI3*, and *TNNC1* probands. *indicates significance

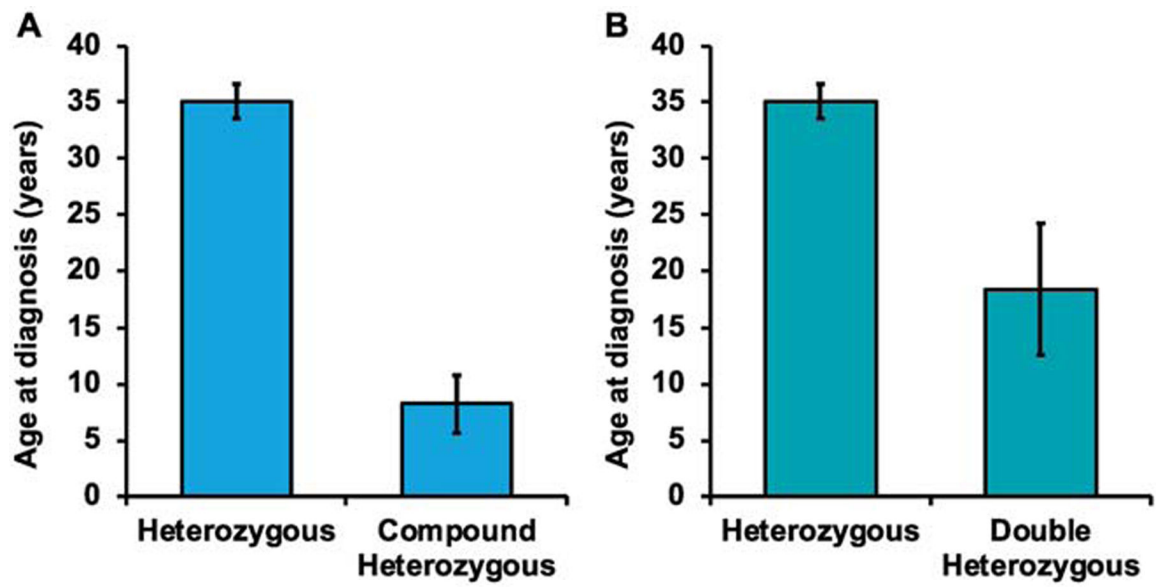


Figure 3: A. Histogram exhibiting distribution of ages of diagnoses in probands with compound heterozygosity and probands with heterozygosity. **B.** Histogram exhibiting distribution of ages of diagnoses in probands with double heterozygosity and probands with heterozygosity. *indicates significance

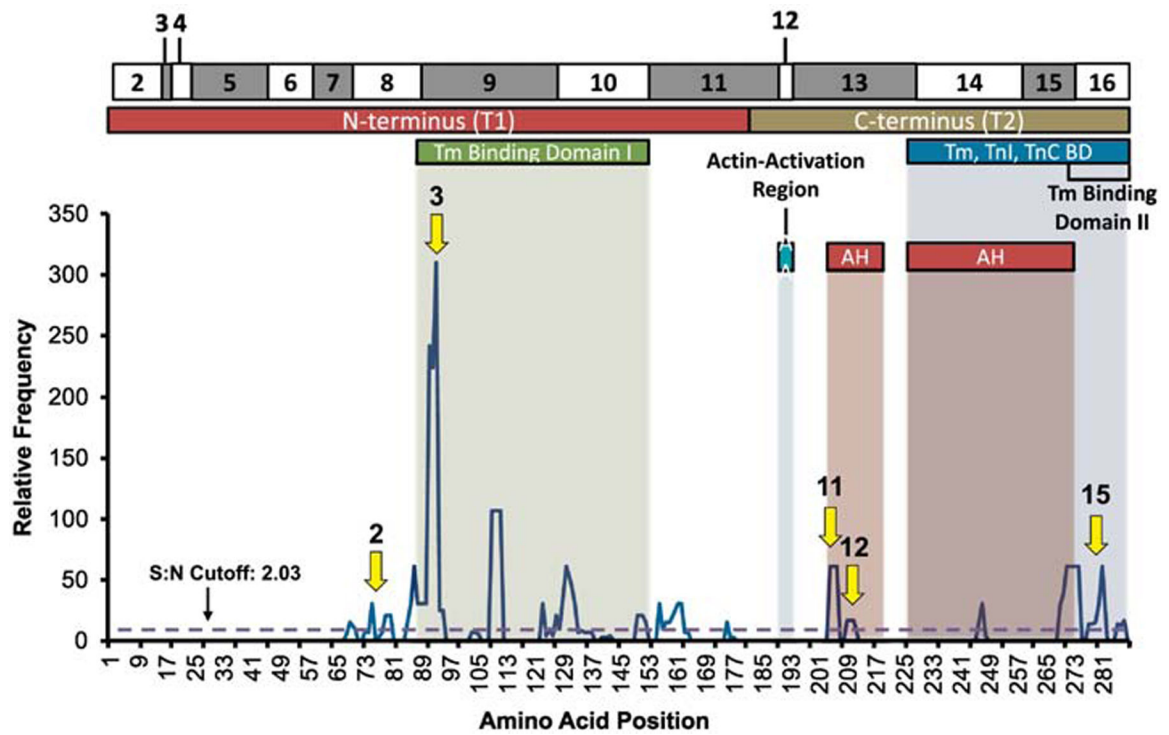


Figure 4:
Signal-to-noise ratio analysis of *TNNT2* probands mapped against TnT amino acid location and domain: N-Terminus, tropomyosin (Tm) binding domains, actin-activation region, the Tm, TnI, and TnC binding domain, and alpha helix (AH) 1 and 2. Arrows point to significant hotspots with outcome relevance.

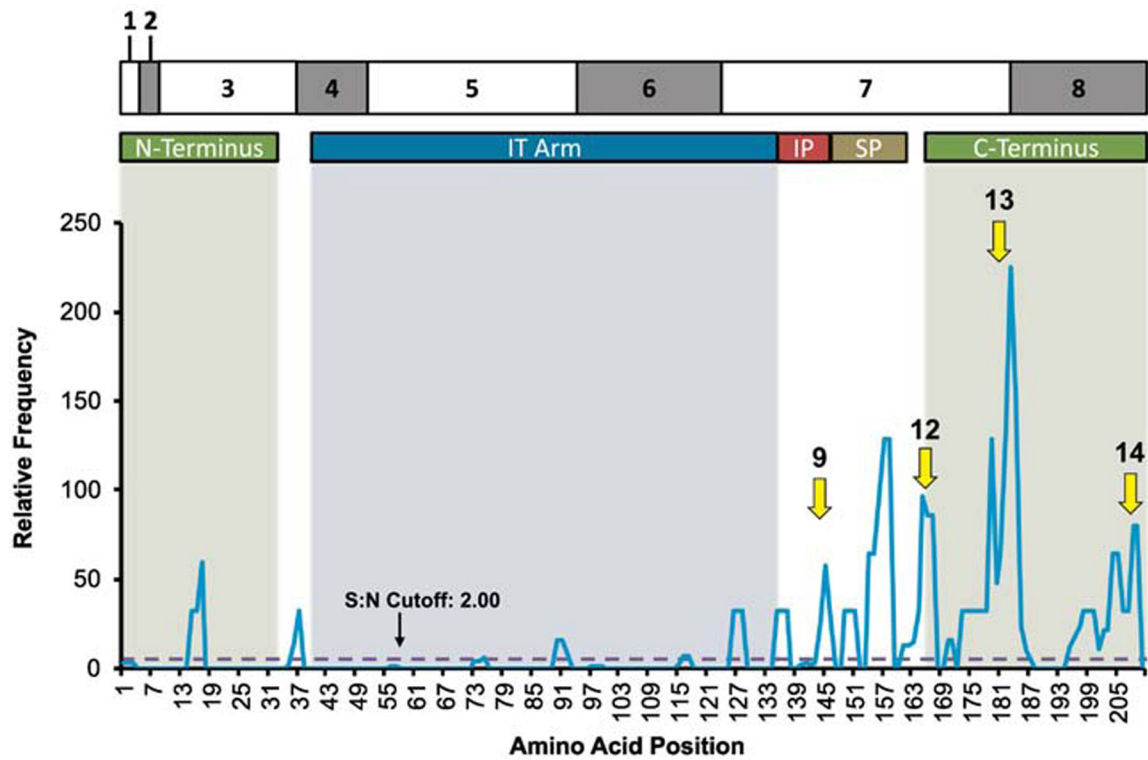


Figure 5: Signal-to-noise ratio analysis of *TNN13* probands mapped against TnI amino acid location and domains: N-terminal extension, I-T arm, inhibitory region (IR), switch-peptide domain (SP), and C-terminus/mobile domain. Arrows point to significant hotspots with outcome relevance.