Discovery of TITIN Gene Truncating Variant Mutations and 5-Year Outcomes in Patients With Nonischemic Dilated Cardiomyopathy

Jeffrey L. Anderson, MD[a,b,*], G. Bryce Christensen, PhD[a,c], Helaman Escobar, MBA[a,c], Benjamin D. Horne, PhD[a,d], Stacey Knight, PhD[a,b], Victoria Jacobs, PhD[a], Kia Afshar, MD[a,b], Virginia B. Hebl, MD[a,b], Joseph B. Muhlestein, MD[a,b], Kirk U. Knowlton, MD[a,b], John F. Carlquist, PhD[a,b], and Lincoln D. Nadauld, MD[a,c]

Genetic factors play an important role in nonischemic dilated cardiomyopathy (NIDC). However, prime opportunities remain for genetic discovery and prognostic understanding. TITIN gene truncating variant mutations (*TTNtv*) are of interest because of their frequent appearance in NIDC series. We sought to discover known and novel *TTNtv* mutations in a NIDC cohort and assess 5-year outcomes. Patients with NIDC entered into the INSPIRE Registry with ≥3 years of follow-up were studied. Whole exome sequencing (WES) was performed using an Illumina Novaseq platform. Genetic analysis used Sentieon software and the GRCh38 human reference genome. Variant calls were annotated with ClinVar. Five-year outcomes were determined by functional assessment and ejection fraction (EF) as recovered (EF ≥50%), persistent (EF 21% to 49%), or progressive (left ventricular assist device, transplant, heart failure [HF] or arrhythmic death, or EF ≤20%). The study comprised 229 NIDC patients (age = 50 ± 15 years, 58% men). *TTNtv*'s were discovered in 27 patients with 22 unique mutations; (7 known, 15 novel). *TTNtv*+ patients more frequently presented with severe NIDC (EF ≤20%) (p = 0.032). By 5-year, outcomes were worse in *TTNtv*+ patients (p = 0.027), and patients less often recovered (11% vs. 30%). Prognosis was similar with known and novel mutations. Nongenetic (e.g., environmental) cocausal risk factors for HF were frequently present, and these factors frequently appeared to act in concert with genetic variants to precipitate clinical HF. In conclusion, our study expands the library of likely pathogenic *TTN* mutations and increases our understanding of their clinical impact in association with other HF risk factors. **(Am J Cardiol 2020;137:97−102)**

Nonischemic dilated cardiomyopathy (NIDC)[1] is believed to involve a spectrum of genetic (familial), nongenetic (environmental), and unknown factors.[2−4] The prevalence of NIDC is estimated to be ≥1 in 250 individuals.[4] It is estimated that familial NIDC may comprise up to 30% to 50% of idiopathic NIDC[2,5,6] and that about 40% of the NIDC patients may have a predisposing genetic basis.[3,4,6,7] However, much of this genetic basis remains unknown. Mutations in the titin gene (*TTN*) are of interest because of their common and consistent appearance in NIDC series.[2,8−10] *TTN* encodes the massive titin protein,[2,9] which plays an important role in skeletal and cardiac muscle development, function, and cell signaling.[8,11,12] The management of NIDC is focused on LV functional evaluation and treatment of heart failure (HF) and arrhythmia-related morbidity/mortality.[13−17] The purpose of this study was to discover the presence of novel as well as known *TTN* truncating variants (*TTNtv*) by whole exome sequencing to determine their prognostic significance and the role of cocausal HF risk factors.

Methods

Study objectives were: (1) To discover the presence of all *TTNtv*'s in a consecutive series of NIDC patients using next-generation whole exome sequencing (WES), (2) to determine their impact on NIDC prognosis, and (3) to assess the prevalence of cocausal (nongenetic) risk factors. We hypothesized that WES would yield additional *TTNtv*'s of prognostic (pathogenic) significance. We specifically tested whether disease presentation and progression in *TTNtv*-positive patients would differ from those of non-*TTNtv*+ NIDC.

The study used a prespecified retrospective observational cohort study design to analyze prospectively collected leukocyte-derived DNA samples linked to electronic medical

[a]Intermountain Medical Center, Intermountain Heart Institute, Salt Lake City, Utah; [b]University of Utah, School of Medicine, Salt Lake City, Utah; [c]Intermountain Precision Genomics Institute, Saint George, Utah; and [d]Division of Cardiovascular Medicine, Department of Medicine, Stanford University School of Medicine, Palo Alto, California. Manuscript received July 24, 2020; revised manuscript received and accepted September 15, 2020.

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*Corresponding author: Tel: 1-801-507-4760.

E-mail address: Jeffreyl.anderson@imail.org (J.L. Anderson).

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records. The study was approved by the Intermountain Healthcare Institutional Review Board.

Consecutive patients with confirmed NIDC presenting to Intermountain Medical Center Specialty Clinics and entered into the INSPIRE DNA and Plasma Bank Registry between January 1999 and September 2016 were studied (N = 229). With Institutional Review Board approval, the INSPIRE Registry has collected blood samples for over 20 years from both inpatients and outpatients being evaluated for cardiovascular diseases who have provided written informed consent to be included in anonymized genetic and biomarker discovery studies.

Patient selection from the INSPIRE Registry participants was limited to those with a primary nonischemic, idiopathic (i.e., potentially genetically determined), dilated cardiomyopathy with a reduced ejection fraction (EF). If environmental or co-morbidity risk factors were present, these were judged by the HF service specialists to be inconclusive or insufficient alone to explain the development of cardiomyopathy.

Specific HF etiologies were excluded, including ischemic cardiomyopathy (assessed by coronary angiography or advanced imaging studies), hypertrophic and hypertensive cardiomyopathies, HF with preserved (≥50%) EF (HFpEF), infiltrative cardiomyopathies (e.g., amyloid, sarcoid), restrictive cardiomyopathies, primary valvular heart disease-related cardiomyopathy, and clearly toxic cardiomyopathies (e.g., in close association with cardiotoxic chemo- or radiation therapy). Post- or peripartum status was not an exclusion, given the shared genetic predisposition, including by *TTN-tv* mutations, with NIDC.

Patients with atrial fibrillation or other tachyarrhythmias, hypertension, diabetes, obesity, mild-moderate or secondary valvular disease (e.g., functional mitral regurgitation), distant chemotherapy, or alcohol or recreational drug use were not excluded if these factors were not considered to be the primary or predominant pathogenic factors or were secondary to or were related to progression of NICD. The strength of these HF cofactors was clinically assessed in individual patients (blinded to genotype) as none/minor, mild/moderate, or moderate/strong, and their impact on *TTNtv* genetic status and outcomes was determined.

Demographic, testing, treatment, and outcomes data of interest were obtained from the INSPIRE Registry[18] and from the Intermountain's electronic medical records database for each unique, qualifying patient. For consistency in reporting and analysis, echocardiography was chosen as the preferred method for left ventricular EF measurement, both at baseline and during follow-up. Angiographic, magnetic resonance, or radionuclide assessment was used when echo measurement was unavailable.

DNA was extracted from blood leukocytes using standard methods and stored until study analysis. Whole exome sequencing was performed on thawed DNA samples by Intermountain Precision Genomics (St. George, UT). DNA samples were normalized to a mass input of 250 or 500 ng before fragmentation to ~400 bp by sonication using the Covaris LE220, followed by purification using AMPure XP Beads (Beckman Coulter). End repair and A-tailing reactions followed by adapter ligation were carried out using the KAPA Hyper Prep kit (Roche) according to the manufacturer's protocol. The resulting NGS libraries were again purified using AMPure XP Beads. Whole exome libraries were then generated using the IDT xGen Exome Research Panel (Integrated DNA Technologies). Fragment sizes for all exome libraries were measured using the Fragment Analyzer High Sensitivity NGS Fragment Analysis Kit (Agilent), and qPCR was performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) using the KAPA Library Quantification Kit (Roche). Sample libraries were pooled by equal mass. Library pools were then diluted to 1.5 nM (to result in a final loading concentration of 300 pM) and denatured using 0.2 N NaOH and quenched with 400 mM Tris-HCl (pH 8.0). Denatured libraries were then loaded onto the NovaSeq 6000 and sequenced using the NovaSeq 6000 S4 Reagent Kit (2 × 150 bp reads). Exome target regions were sequenced at a mean depth of 93x.

Alignment and variant calling were performed with Sentieon analysis software version 201704.03 (Sentieon, Inc.) based on the GRCh38 human reference genome. Variants were called with the Sentieon "Haplotyper" method. Variants were annotated using Golden Helix VarSeq v2.1.0, based on annotations from ClinVar (June 2019 update), ExAC variant frequencies 0.3 v2 (BROAD Institute), and gnomAD exomes variant frequencies 2.0.1 (BROAD Institute). *TTNtvs* were defined as nonsense variants and/or frameshift variants that introduced a premature termination codon in the *TTN* transcript. *TTNtv* variants were designated as novel if they did not appear in the gnomAD or ExAC variant frequency databases and were not classified in ClinVar. All *TTNtv* variants were validated by reprocessing the sample with the Illumina Dragen analysis pipeline, version 3.2.2. Dragen results matched Sentieon results for all *TTNtv's*.

Clinical status of study patients was assessed at initial presentation and at years 1, 5, and last follow-up or until cardiac transplantation or death by review of individual medical records by an experienced study investigator (JLA) blinded to genotype. The closest encounter to 5 years (±2 years) was chosen to assess the primary clinical outcome. Disease severity at presentation was defined by clinical functional assessment and left ventricular EF (LVEF), wherever available, and categorized as mild (LVEF 40% to 49%), moderate (LVEF 21% to 39%), or severe (EF ≤20%). Disease status during follow-up was categorized by clinical assessment and LVEF, wherever available, as recovered (LVEF ≥50%), persistent (LVEF 21% to 49%), or progressive (left ventricular assist device [LVAD], transplant, VT/VF, HF, death, or LVEF ≤20%).

The primary hypothesis was that the percentage of 5-year severe NIDC would be different between *TTNtv*-positive status and *TTNtv*-negative status. We also examined the association between severity of NIDC at time of presentation and *TTNtv* status. We further tested whether presentation and subsequent prognosis differed between novel and reported *TTNtv* variants.

Demographics of the study population and location and type of genetic variants are presented descriptively. Fisher's exact testing and ordered exact chi-square testing were used to assess differences in disease category at presentation and the 5-year primary follow-up interval between *TTNtv*-positive and -negative NIDC patients.

Results

A total of 229 NIDC patients in the INSPIRE registry qualified for the study. Demographics are shown in Table 1. Age averaged 50 ± 15 years, 58% were men, and 86% were white. At presentation, LVEF averaged 26 ± 10%.

A total of 22 *TTN* mutations resulting in a TTN truncation (*TTNtv*) were found in 27 patients (Table 2). Among the 15 patients with a documented family history of cardiomyopathy, 4 (27%) were *TTNtv*+.

Of these 22 discovered mutations, half (n = 11) were stop-gain mutations, and the other half (n = 11) were frameshifts that resulted in a truncated protein. A total of 7 (32%) of the mutations were found in ClinVar; 1 was reported as pathogenic, 5 where reported as likely pathogenic, and 1 was reported as a variant of uncertain significance (VUS). A total of 15 were novel (not reported in ClinVar) and were found in 18 patients. Most of the TTNtv mutations were found on the A-Band (n = 17, 77%) followed by in the I-Band (n = 3, 14%) and the M-Band (n = 2, 9%) (Figure 1). Most of novel *TTNtv's* were found on the A-band (n = 13, 87%). Of the other 2 novel mutations, 1 was found on the I-band within 107 kb of a *TTNtv* mutation classified as likely pathogenic and also within 28 kb of a VUS. The other mutation was on the M-band within 2 kb of a *TTNtv* mutation classified as pathogenic.

Table 1
Demographics and selected baseline variables of study population

Baseline demographics	(N = 229)
Age (years)	50 ± 15
Men	134 (58.5%)
White	197 (86.0%)
Diabetes mellitus	36 (15.7%)
Atrial fibrillation	58 (25.3%)
Hypertension	56 (24.5%)
Current smoker	10 (4.4%)
Ejection fraction (%) on presentation	26 ± 10

Demographic variables did not significantly differ for patients with *TTNtv* mutations and those without although *TTNtv*+ patients tended to be younger, more frequently had a positive family history, and had a lower presenting EF (see Supplementary Table 1).

Table 2
TTN heterozygous truncating variant, loss-of-function mutations

Position	Variant Type	ClinVar Classification	Band	HGVS c. variant	HGVS p. variation
178531739	Frameshift	Novel	M-Band	c.104861_104876del	p.Pro34954Hisfs*5
178534092	Stop-Gain	Pathogenic	M-Band	c.102523C>T	p.Arg34175Ter
178536358	Stop-Gain	Novel	A-Band	c.100389C>A	p.Tyr33463Ter
178537173	Stop-Gain	Novel	A-Band	c.99936G>A	p.Trp33312Ter
178539926	Frameshift	Novel	A-Band	c.98139dupT	p.Ile32714Tyrfs*24
178547845	Stop-Gain	Likely Pathogenic	A-Band	c.93781C>T	p.Arg31261Ter
178549328	Stop-Gain	Novel	A-Band	c.92298G>A	p.Trp30766Ter
178561543	Frameshift	Novel	A-Band	c.84586_84589delAGAA	p.Arg28196Alafs*18
178565120	Stop-Gain	Likely Pathogenic	A-Band	c.80997_81012del	p.Tyr26999Terfs
178567223	Frameshift	Novel	A-Band	c.78909delA	p.Lys26303Asnfs*4
178570543	Stop-Gain	Novel	A-Band	c.75589delG	p.Val25197Terfs
178574312	Frameshift	Novel	A-Band	c.71819_71820delCA	p.Thr23940Serfs*4
178574443	Frameshift	Novel	A-Band	c.71689delT	p.Tyr23897Metfs*5
178575909	Frameshift	Likely Pathogenic	A-Band	c.70210_70223delGAATGTAACAGATAinsTTTACTCTTC	p.Glu14339Phefs*11
178580521	Frameshift	Novel	A-Band	c.66858dupA	p.Gly22287Argfs*11
178585291	Stop-Gain	Likely Pathogenic	A-Band	c.64453C>T	p.Arg21485Ter
178586614	Frameshift	Novel	A-Band	c.64287delT	p.Gly21430Alafs*2
178588715	Stop-Gain	Novel	A-Band	c.63010G>T	p.Glu21004Ter
178612534	Frameshift	Novel	A-Band	c.49991delC	p.Thr16664Lysfs*7
178632294	Stop-Gain	Likely Pathogenic	I-Band	c.43600C>T	p.Gln14534Ter
178739394	Frameshift	Novel	I-Band	c.13838_13839delTG	p.Val4613Glyfs*5
178767782	Stop-Gain	VUS	I-Band	c.9448C>T	p.Arg3150Ter

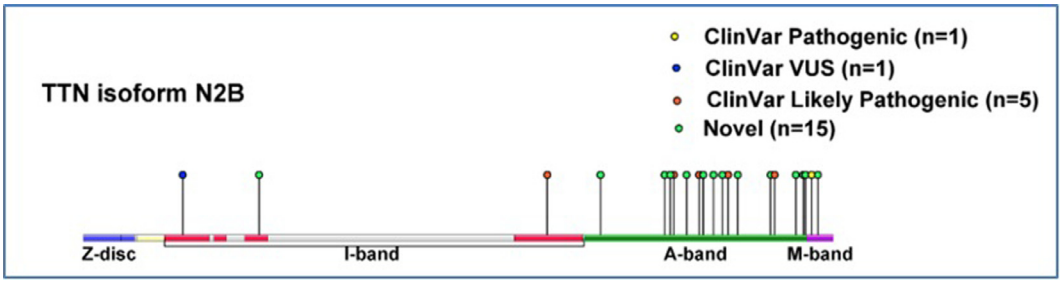

Figure 1. Genomic position of novel and reported TTNtv mutations on the TTN cardiac isoform N2B with protein bands as indicated.

Table 3
Initial presentation and 5-year outcomes by TITIN truncating mutation (*TTNtv*) status in the NIDC cohort*

Presentation heart failure status	TTNtv+ (n = 25)	TTNtv- (n = 194)	p Value† 0.032
Severe	20 (80%)	112 (58%)	
Mild/Moderate	5 (20%)	82 (42%)	
Five-year heart failure status	(N = 27)	(N = 202)	0.027
Progressive¶	11 (41%)	51 (25%)	
Persistent§	13 (48%)	91 (45%)	
Recovered‡	3 (11%)	60 (30%)	

* Note: Includes 10 patients with novel and 7 with known *TTNtv* mutations. Severity at presentation was not documented for 10 of the TTN- patients.

† p Values based on ordered exact chi-square test.

‡ Ejection fraction (EF) averaged 58.9 ± 7.1% and did not differ between *TTNtv* groups (52.3 ± 1.5 [3], 58.4 ± 7.2 [41], p = 0.16).

§ EF averaged 36.3 ± 7.9% and did not differ between *TTNtv* groups (39.7 ± 7.1% [13], 35.7 ± 7.9% [72], p = 0.10).

¶ Only 1 patient was categorized by EF (=13%); see S2 Table.

An additional 34 unique samples were found to carry nontruncating variants that were classified as VUS in ClinVar. As the pathogenicity of these variants is uncertain or unlikely, patients with these nontruncating variants were excluded from the comparative and outcomes analyses.

Concomitant HF risk factors and relevant co-morbidities were common overall in our NICM population (1.44/patient) and were statistically similar individually and combined in the 2 groups (1.4, 1.7 per *TTNtv*-, *TTNtv*+ patients, respectively) (Supplementary Table 1). The frequency of *TTNtv* mutations also was statistically similar in patients whose concomitant environmental or co-morbidity risk factors were assessed as having none/minor (23/185 = 12.4%) and mild/moderate (4/31 = 12.9%) impact. No *TTNtv* mutations were found in the 13 patients with moderate/strong concomitant nongenetic risk factors, but this difference was not significant (p = 0.37, Fisher's exact test).

Severe NIDC at initial medical presentation (LVEF ≤20%) was noted in 219 of the subjects, 20 of 25 (80%) *TTNtv*+ versus 112 of 194 (58%) *TTNtv*- patients (p = 0.032). By 5 years, there was a worse outcome in *TTNtv*+ patients (p = 0.027), with NIDC less often recovering (11%, n = 3 vs. 30%, n = 60; p = 0.042) in *TTNtv*+ patients compared with *TTNtv*- patients (Table 3). Prognosis was similar with known and novel mutations (Table 4).

Table 4
Initial presentation and 5-year outcomes in 17 TITIN truncating mutation (*TTNtv*)+ patients by novelty

Variable	TTNtv novel	TTNtv known	Unadjusted p Value*
Presentation heart Failure status	(n = 16)	(n = 9)	0.12
Severe	11 (80%)	9 (100%)	
Mild/Moderate	5 (20%)	0	
Five-year heart failure status	n = 18	n = 9	1.0
Progressive/Persistent	16 (89%)	8 (89%)	
Recovery	2 (11%)	1 (11%)	

* p Values based on Fisher exact tests.

Of *TTNtv*+ patients who showed HF progression over 5 years, 3 suffered cardiovascular death (i.e., HF death in 2, sudden death in 1), and 1 had a resuscitated cardiac arrest. The distribution of progressive outcome events was similar in *TTNtv*- patients (Supplementary Table 2) although their overall frequency tended to be lower (25% vs. 41%; p = 0.088). Consistent with more advanced disease and worse outcomes, *TTNtv*+ patients tended to receive more advanced therapies: 26% (n = 7) of *TTNtv*+ patients progressed to cardiac transplantation versus 15% (n = 31) of *TTNtv*- patients (p = 0.10) (Supplementary Table 2). Supplementary Table 3 shows the individual frequency of cardiac implantable cardioverter defibrillators (ICDs) and left ventricular assist devices (LVADs) that patients in the 2 genotype-determined groups received during the course of treatment. ICDs were used in the majority of patients across the 2 groups (65%) although they tended to be more frequently implanted in *TTNtv*+ patients (p = 0.12). LVAD implantation also was more frequently required in the *TTNtv*+ group, usually as a prelude to cardiac transplantation (26% vs. 10%, p = 0.015).

Discussion

In this moderately large, representative cohort of NIDC patients, the use of WES permitted the discovery of 27 *TTNtv's* in 22 patients, including 15 novel mutations in 18 patients. These novel mutations were located primarily within a narrow region of chromosome 2 in proximity to known pathogenic mutations. *TTNtv*+ patients presented more frequently with severe cardiomyopathy, showed a worse 5-year prognosis, and less often recovered. The poor prognosis of patients with one of the 15 novel mutations was similar to that of those with one of the 7 known mutations, suggesting pathogenic similarity. Given the recognized high likelihood of *TTNtv's* to be pathogenic, the proximity of the novel to known mutations, and their similarly malignant course, we propose these newly discovered *TTNtv* mutations to be classified as likely pathogenic. *TTNtv* mutations frequently occurred in the setting of environmental risk factors and co-morbidities, suggesting that the latter frequently combine with genetic susceptibility to precipitate HF.

TTN is of interest to NIDC because *TTNtv* mutations have consistently and frequently been found in NIDC series[2,8–10] including in up to 20% to 25% of familial cases.[2,8–10,19] *TTN* encodes the massive titin protein, which contains ~35,000 amino acids.[2,9] As a constituent of skeletal and cardiac muscle sarcomeres, titin plays a key role in cellular development, function, and signaling.[8,11,12] The large and complex *TTN* gene encodes 364 exons, and these undergo extensive splicing to produce many isoforms. *TTN* variants related to NIDC are found primarily in highly expressed exons of the cardiac titin isoforms N2B and N2BA, which are most frequently located in the A-band and are highly likely to be pathogenic.[2,10,20] The *TTN* variants found in most other regions are correlated with generally low penetrance, but combined with a second, for example, environmental factor or co-morbidity, they may coprecipitate HF.[21]

Support for a pathogenic role of *TTNtv's* is provided by pluripotent stem cell (iPSC) technology, which has revealed dysfunctional cardiomyocytes generated from NIDC patients.[9,11,12] Additionally, LV wall fibrosis, ventricular and atrial tachyarrhythmias, and earlier or more likely implantation of LV assist devices and listing for cardiac transplantation have been reported for *TTNtv*+ patients.[11,22] However, these proposals are based on limited and conflicting observations on clinical outcomes.[23]

In a seminal 2012 report,[9] *TTNtv's* were found in 27% of NIDC, 1% of hypertrophic cardiomyopathy, and 3% of control patients. *TTNtv's* were located predominantly in the sarcomere A-band and cosegregated with familial cases with high penetrance after age 40. Outcomes did not differ by *TTNtv* status, but men presented at an earlier age. The 1000 Genomes Project found a much lower prevalence of A-band *TTNtv's* in the general population, that is, 1 in 500.[21] The large Exome Aggregation Consortium[21] reported a prevalence of 12.4% *TTNtv's* in the NIDC population (n = 1788) compared with 0.36% in the reference population (n >60,000) and estimated the probability of pathogenicity of *TTNtv* in the A-band and I/A-band junction to be 97.8%. In contrast, *TTN* missense and nonframeshifting insertions/deletions did not predict pathogenicity.[24,25] (Based on these reports, we discounted the significance of nontruncating variants, including a single controversial missense variant noted in 7 of our NIDC patients.) Adding to HF disposition, Tayal et al reported that *TTNtv's* independently predicted early arrhythmias (i.e., atrial fibrillation, nonsustained and sustained ventricular tachycardia) in patients with *TTNtv*+ NIDC.[22] In contrast to other reports and our experience of equivalent or worse prognosis, Jansweijer et al published a cohort of *TTNtv*+ patients with a milder and more treatment-responsive form of NIDC.[23]

In our study, as in the more recent published series in broader populations, we found a low but important frequency of *TTNtv's* among general NIDC patients and a more than doubling in those with a family history of cardiomyopathy. Also, in contrast to Jansweijer, our experience was consistent with those reporting at least as severe or more severe outcomes. Our report also adds to previously annotated mutations with 15 novel *TTNtv's* with strong direct and contextual evidence of pathogenicity.

Strengths of our study include the use of next-generation WES, which enables discovery of mutations missed by targeted genetic approaches. Our consecutive series broadly represents NIDC patients presenting for specialty care, and our long-standing electronic medical record system has allowed for long-term follow-up. Limitations include the referral nature of patients sent to our HF specialty clinics, which likely biases the cohort to more severe cases. Also, there was some loss of initial and follow-up information on the patient subset whose initial and/or long-term care was outside of the Intermountain Healthcare network. For this reason, we were unable to determine overall frequency of HF hospitalizations. Co-morbidities may have been undercounted in some patients due to limitations in the clinical records available for review. Although moderately large overall, the study represents a relatively small number of *TTNtv*+ patients on which to base comparisons of outcomes. Nevertheless, a high percentage (two-thirds) of discovered *TTNtv* mutations were found to be novel, and these represent a noteworthy contribution to future NIDC diagnostics and, potentially, to therapeutics.

Credit Author Statement

Jeffrey L. Anderson: Conceptualization, formal analysis, writing-original draft, writing-review and editing, supervision, project administration. *G. Bryce Christensen:* Conceptualization, investigation, writing-review and editing. *Helaman Escobar:* Investigation, writing-review and editing. *Benjamin D. Horne:* Conceptualization, formal analysis, data curation, writing-review and editing. *Stacey Knight:* Conceptualization, formal analysis, data curation, writing-review and editing. *Victoria Jacobs:* Investigation, writing-review and editing. *Kia Afshar:* Investigation, writing-review and editing. *Virginia B. Hebl:* Investigation, writing-review and editing. *Joseph B. Muhlestein:* Investigation, writing-review and editing. *Kirk U. Knowlton:* Investigation, writing-review and editing. *John F. Carlquist:* Investigation, writing-review and editing. *Lincoln D. Nadauld:* Formal analysis, investigation, writing-review and editing.

Disclosures

None.

Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.amjcard.2020.09.026.

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