

ORIGINAL ARTICLE

Amino Acid-Level Signal-to-Noise Analysis Aids in Pathogenicity Prediction of Incidentally Identified *TTN*-Encoded Titin Truncating Variants

Patrick S. Connell¹, MD, PhD*; Amy M. Berkman¹, MD*; BriAnna M. Souder, DO, MS; Elisa J. Pirozzi¹, BS; Julia J. Lovin¹, MD; Jill A. Rosenfeld¹, MS; Pengfei Liu, PhD; Hari Tunuguntla¹, MD; Hugh D. Allen¹, MD; Susan W. Denfield, MD; Jeffrey J. Kim¹, MD; Andrew P. Landstrom¹, MD, PhD

BACKGROUND: *TTN*, the largest gene in the human body, encodes TTN (titin), a protein that plays key structural, developmental, and regulatory roles in skeletal and cardiac muscle. Variants in *TTN*, particularly truncating variants (TTNtvs), have been implicated in the pathogenicity of cardiomyopathy. Despite this link, there is also a high burden of TTNtvs in the ostensibly healthy general population. This complicates the diagnostic interpretation of incidentally identified TTNtvs, which are of increasing abundance given expanding clinical exome sequencing.

METHODS: Incidentally identified TTNtvs were obtained from a large referral database of clinical exome sequencing (Baylor Genetics) and compared with rare population variants from genome aggregation database and cardiomyopathy-associated variants from cohort studies in the literature. A subset of TTNtv-positive children evaluated for cardiomyopathy at Texas Children's Hospital was retrospectively reviewed for clinical features of cardiomyopathy. Amino acid-level signal-to-noise analysis was performed.

RESULTS: Pathological hotspots were identified within the A-band and N-terminal I-band that closely correlated with regions of high percent-spliced in of exons. Incidental TTNtvs and population TTNtvs did not localize to these regions. Variants were reclassified based on current American College of Medical Genetics and Genomics criteria with incorporation of signal-to-noise analysis among Texas Children's Hospital cases. Those reclassified as likely pathogenic or pathogenic were more likely to have evidence of cardiomyopathy on echocardiography than those reclassified as variants of unknown significance.

CONCLUSIONS: Incidentally found TTNtvs are common among clinical exome sequencing referrals. Pathological hotspots within the A-band of *TTN* may be informative in determining variant pathogenicity when incorporated into current American College of Medical Genetics and Genomics guidelines.

Key Words: cardiomyopathies ■ exome ■ genetic testing ■ incidental findings ■ population

Recent advances in genetic sequencing technologies provide unprecedented research and clinical diagnosis opportunities. Exome sequencing (ES) is a cost-effective tool that enables sequencing of all protein-coding regions of the genome thus facilitating detection of both coding and splice-site genetic variants. It has the unique benefit of allowing the accurate

diagnosis of individuals with Mendelian disorders that may have atypical presentations or require extensive and expensive lab testing for diagnostic confirmation.¹ While ES is a powerful diagnostic tool, it has also led to the dramatic increase in identification of incidentally identified variants, also known as secondary findings, which are associated with potentially life-threatening disease yet

Correspondence to: Andrew P. Landstrom, MD, PhD, Duke University Medical Center, Box 2652, Durham, NC 27710. Email andrew.landstrom@duke.edu

*Drs Connell and Berkman are joint first authors.

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Nonstandard Abbreviations and Acronyms

ACMG	American College of Medical Genetics and Genomics
DCM	dilated cardiomyopathy
ES	exome sequencing
gnomAD	genome aggregation database
LP/P	likely pathogenic/pathogenic
PSI	percent-spliced in
S:N	signal-to-noise
TTN	titin
TTNts	truncating variants in TTN
VUS	variant(s) of unknown significance

were not suspected at the time of genetic testing. The American College of Medical Genetics and Genomics (ACMG) recognizes that new sequencing platforms have led to a high burden of these incidental variants and provides guidelines for assessing the likelihood of pathogenicity to determine whether variants should be reported during return of results.² Fifty-nine genes (ACMG-59) have been designated reportable should an incidental variant be identified if that variant is determined to be likely pathogenic or pathogenic (LP/P). Despite this guidance, discerning the pathogenicity of variants, particularly those related to cardiovascular disease, remains a challenge due to reduced penetrance of disease and variable expressivity.

TTN, the largest gene in the human body, encodes cardiac TTN (titin), a protein that is heavily involved in the functioning of sarcomeres, the basic contracting units of both skeletal and cardiac muscle cells. Given its key roles in the structure, development, and regulation of cardiac sarcomeres, variants in *TTN* have been implicated in the pathogenicity of highly morbid cardiomyopathies. Indeed, *TTN* has been found to be one of the most commonly mutated genes among probands with dilated cardiomyopathy (DCM) who underwent genetic testing and is a rare cause of hypertrophic cardiomyopathy.³ Specifically, heterozygous truncating variants in *TTN* (TTNts), those that lead to early termination of translation, have been implicated in familial cardiomyopathies in both pediatric and adult populations.⁴

Cardiomyopathies are primary diseases of the ventricular myocardium that are not caused by congenital heart disease or abnormal loading conditions and are classified by morphology and physiology.⁵⁻⁷ In the pediatric population, DCM and hypertrophic cardiomyopathy are the most common subtypes, and left ventricular non-compaction cardiomyopathy is less common. Despite the clear link between TTNts and cardiomyopathy, there is a high burden of TTNts among the ostensibly healthy population. Specifically, TTNts have also been

identified in up to 3% of control individuals who had no clinical evidence of cardiomyopathy,⁸ which complicates the diagnostic evaluation of TTNts, particularly when identified incidentally. Previous studies that identified the location of pathological TTNts have found an overrepresentation of pathological variants in the A-band region and a lack of these variants in the M-band and Z-disk regions of *TTN*.^{9,10} Furthermore, given the high degree of splice variability in the transcription of *TTN*, it is being increasingly established that TTNts localizing to exons that are more often spliced, or have a larger percent-spliced in (PSI), are more likely to be associated with development of disease. Given the localization of both disease and population-based variation across the gene, and the increasing burden of incidentally identified TTNts, additional methods to clarify variant pathogenicity are needed. This is particularly important as variants in *TTN* are not currently included in the ACMG list of 59 genes to be reported as incidental findings, whereas other more rare variants linked with cardiomyopathy are included, likely due to this lack of clarity in pathogenicity in TTNts.¹¹ With a better understanding of pathological versus nonpathological variation in *TTN*, inclusion onto this list of reportable findings would be possible.

In the current study, we used amino acid-level signal-to-noise (S:N) and PSI analysis to compare TTNts to identify discrete mutation hotspots within *TTN*. We then used this tool to predict pathogenicity of TTNts identified incidentally during clinical ES testing. Among cases that ultimately underwent evaluation, we found that S:N hotspot analysis incorporation into 2015 ACMG guidelines yielded an increased frequency of pediatric-onset cardiomyopathy when the variants were designated as likely pathogenic versus uncertain significance (VUS).² We conclude that incidental TTNts are potentially commonplace, and S:N analysis may refine the diagnostic utility of the ACMG guidelines.

METHODS

Study methods can be found in the [Data Supplement](#). This research study was approved by the Baylor College of Medicine and Duke University Hospital System Institutional Review Boards. The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

Burden of TTNts Among Clinical ES Referrals

To determine the spectrum and frequency of incidentally identified TTNts among clinical ES testing, we identified these variants in a large clinical ES referral cohort. Figure 1 shows cohort derivation for all cohorts. There were 7938 unrelated clinical ES referrals that met inclusion/exclusion criteria. The demographics of the clinical ES

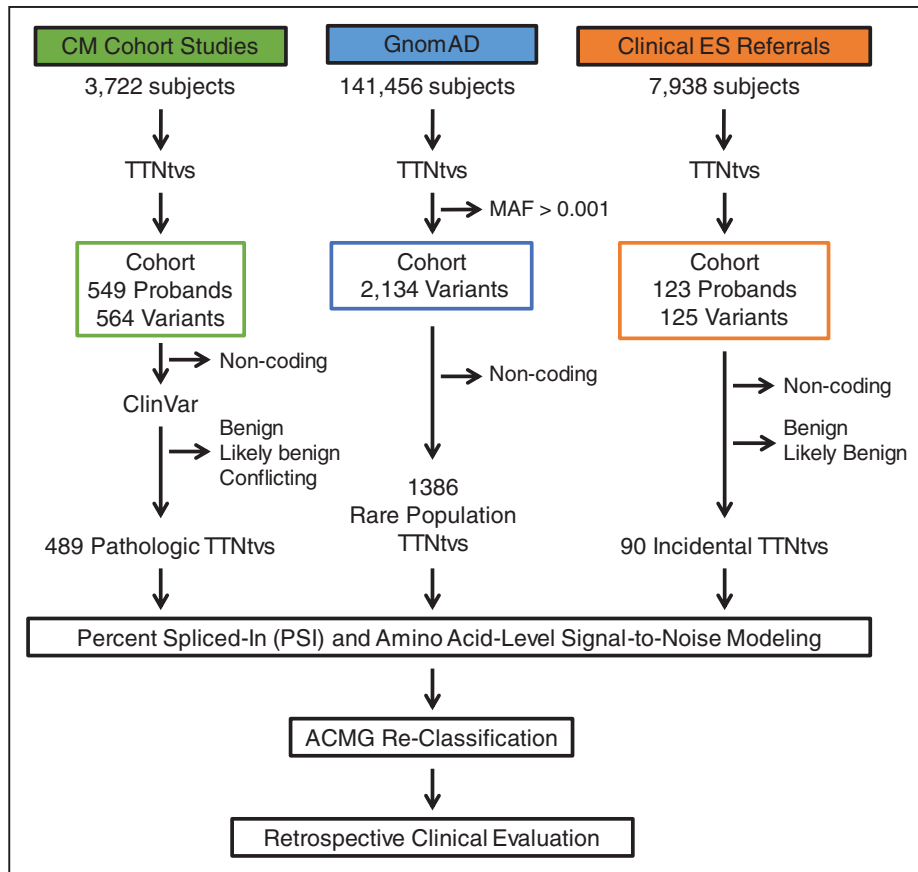


Figure 1. Summary of cohorts and analyses performed.

Flowchart diagramming study design including inclusionary and exclusionary criteria for percent-spliced in (PSI) and amino acid-level signal-to-noise (S:N) modeling and American College of Medical Genetics and Genomics (ACMG) reclassification for *TTN*-encoded titin truncating variants (TTNts). Retrospective clinical evaluation was then conducted on a subset of patients seen at Texas Children's Hospital (TCH). CM indicates cardiomyopathy; ES, exome sequencing; gnomAD, genome aggregation database; and MAF, minor allele frequency.

cohort, and variant positive individuals within the cohort, are detailed in Table I in the [Data Supplement](#). Out of these referrals, 123 probands were found to carry 125 unique TTNts giving a prevalence of 1.6% among clinical ES referrals. Among these 125 variants, 96 (1.2% of all variants found among ES referrals) were assigned LP/P assessment at time of genetic test reporting and 27 (0.3% of referrals) were VUSs. Ninety of these variants were incidental TTNts with valid positions within the meta transcript and were carried forward for additional analysis.

To determine the diagnostic value of these variants for monogenic disease, we established a cardiomyopathy-associated cohort and a rare population-based variant cohort for comparison. The cardiomyopathy cohort, including 3722 patients with cardiomyopathy, was derived from 17 proband-based studies (Table II in the [Data Supplement](#)), confirmed as LP/P by ClinVar, and included 549 probands with 564 TTNts, of which 489 were classified by ClinVar as pathogenic. This gave an overall prevalence of 15% and a cumulative minor allele frequency of 0.076 among pathological cases of cardiomyopathy. The rare population-based cohort was derived

from the genome aggregation database (gnomAD) database. From 141 456 subjects in gnomAD, 2134 TTNts were identified to form this cohort. This gave a cumulative minor allele frequency of 0.008. Of these, 1386 were rare population TTNts, excluding splice-site variants. The yield of TTNts in these cohorts is summarized in Figure 2. A summary of the ES and pathological cohort variants is available in Tables I and II in the [Data Supplement](#). Taken together, these findings show that incidentally identified TTNts are found in similar proportions in ES referrals as are found in the general population while cardiomyopathy-associated TTNts had a significantly higher yield of 15% among cardiomyopathy patients. There were also significant differences in the proportional makeup of variants in our 3 cohorts (Figure 2C, χ^2 test, $P < 0.001$). The cardiomyopathy cohort demonstrated 44% stop gained, 13% splice-site, and 43% frameshift variants, the ES cohort demonstrated 29% stop gained, 27% splice-site, and 44% frameshift variants, while the gnomAD cohort demonstrated 31% stop gained, 35% splice-site, and 34% frameshift variants. Therefore, the burden of variants among the incidentally identified ES cohort was more similar to the rare population-based

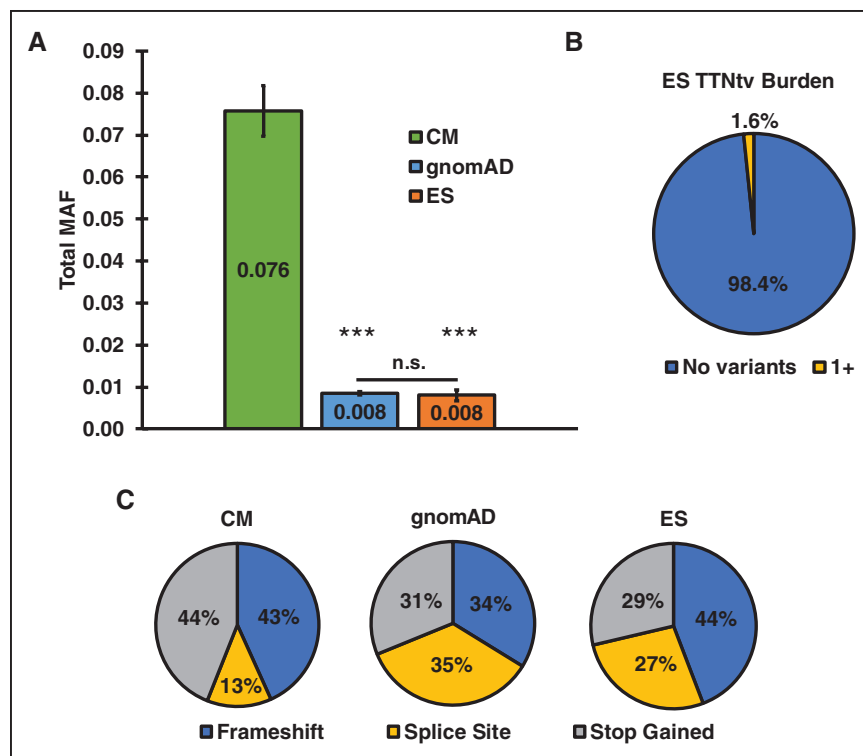


Figure 2. The yield of *TTN*-encoded titin truncating variants (TTNts) among cardiomyopathy cases, genome aggregation database (gnomAD), and exome sequencing (ES).

A, A bar graph representing the minor allele frequency (MAF) of TTNts found in our cardiomyopathy (CM; green), gnomAD (blue), and ES (orange) cohorts. No significant difference was found between gnomAD and ES cohorts. $***P=0.00001$. **B**, A pie chart demonstrating the burden of TTNtv found in the ES cohort (blue=no variants and orange=1+ variant). **C**, Pie charts demonstrating the relative proportion of frameshift (blue), splice-site (yellow), and stop gained (gray) variants among CM, gnomAD, and ES cohorts.

gnomAD cohort, while there was a distinctly higher burden with a different underlying composition among the pathogenic cardiomyopathy cohort.

Amino Acid-Level Pathogenicity Probability Analysis

Given the relatively high yield of incidentally identified variants among clinical ES referrals, we next attempted to develop a method to determine the diagnostic weight of these variants. We have previously shown that amino acid-level S:N calculations, comparing frequencies of disease-associated variants with population-based variants at individual amino acid positions, can differentiate between incidentally identified and pathological variants based on primary sequence location in genes in cardiac channelopathic disease.^{12,13} Thus, we next applied amino acid-level S:N analysis and correlated with PSI analysis, a measurement of how efficiently sequences of interest are spliced in to transcripts within healthy individuals (Figure 3). Overall, the gnomAD cohort variants localized to 811 unique amino acid positions distributed throughout the primary sequence of TTN. When normalized against the overall frequency of rare TTNts found in the gnomAD cohort, we found a marked difference in the S:N values between ES and pathological variants as well as the location of the highest S:N values seen among the 2 cohorts. While the highest peak S:N value for the cardiomyopathy cohort was located in the I-band (peak S:N=333), near the A-band, the majority of high S:N values (peak S:N=277–200) were located

heterogeneously throughout the A-band (Figure 3A and 3B). There were also regions of relatively low S:N values for the cardiomyopathy cohort located throughout the A-band, particularly in positions $\approx 16\,200$ to $\approx 16\,400$, $\approx 22\,400$ to $\approx 23\,200$, $\approx 29\,300$ to $\approx 30\,000$, and $\approx 34\,000$ to $35\,000$. Of note, while smaller peaks are distributed in the M-band (peak S:N=200–117), Z-band (peak S:N=33–25), and the near-Z region (peak S:N=83–67), there is a relative paucity of high S:N values in the cardiomyopathy cohort in the majority of the I-band, with the exception of the previously noted maximum peak near the I-band, A-band junction. The ES cohort has comparatively smaller S:N values, with heterogeneous spread throughout the protein (peak S:N=31–7 outside the I-band), with noted minimum values throughout locations $\approx 15\,000$ to $\approx 20\,000$ in the A-band (peak S:N=0), with a maximum peak located in the mid I-band (peak S:N=43). The per position average S:N for the cardiomyopathy case-cohort variants was 42.3 ± 2.4 compared with 4.0 ± 0.8 in the clinical ES referral cohort ($P < 0.05$), resulting in a yield that was 10-fold higher in the cardiomyopathy cohort compared with the clinical ES referral variants (Figure 3D). Additionally, TTNts found in the cardiomyopathy cohort were more likely to be localized to regions with higher PSI ($98.6 \pm 0.45\%$) compared with variants found in the gnomAD ($65.2 \pm 1.17\%$) and clinical ES referral ($57.8 \pm 4.8\%$) cohorts (Figure 3E). Taken together, this shows that pathological variants exhibit significantly higher frequency measured with S:N analysis and PSI, compared with incidental variants identified in the clinical ES referral cohort ($P < 0.05$).

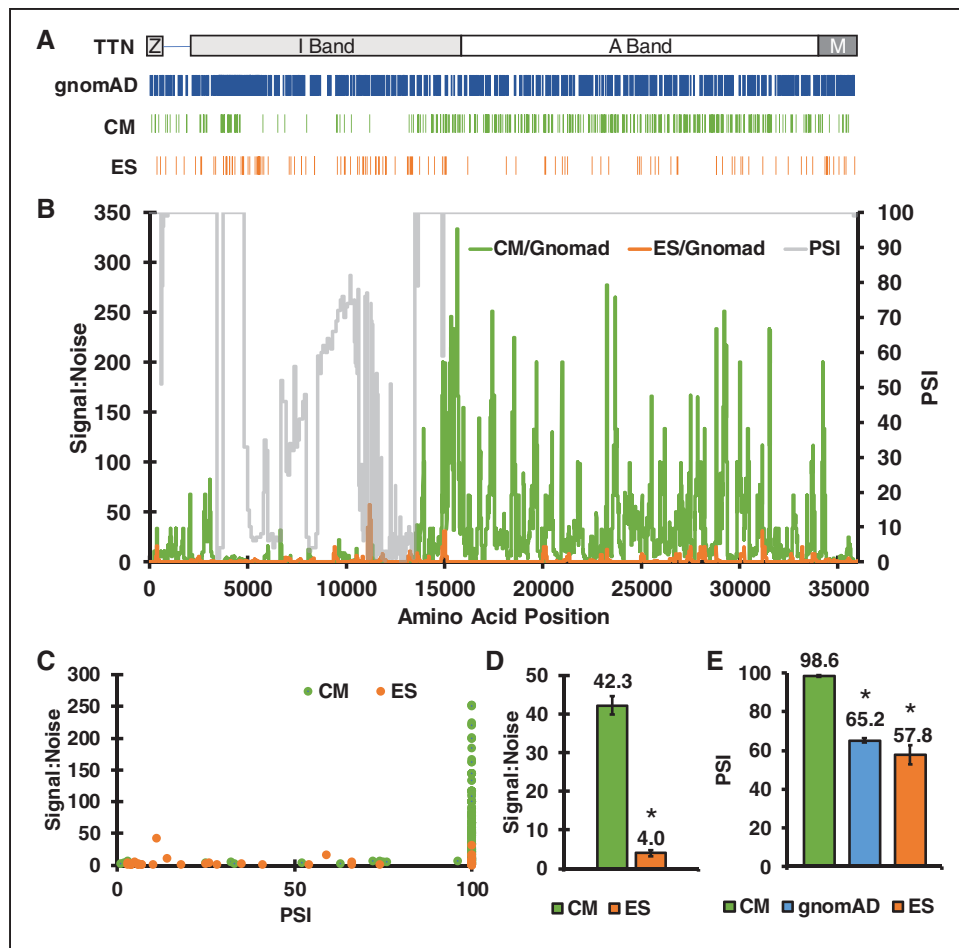


Figure 3. Cardiomyopathy (CM) variants have large signal-to-noise (S:N), large percent-spliced in (PSI) while exome sequencing (ES) variants have low S:N, low PSI.

A, Location of affected amino acid for each variant in the 3 cohorts. Corresponding linear topology of the TTN (titin) protein including the Z-disk, I-band, A-band, and M-band. **B**, Rolling average of the S:N of the nearest 200 variants of the CM (green) and ES (yellow) cohorts compared with genome aggregation database (gnomAD; orange) cohort plotted as a rolling average at each amino acid position along the length of TTN. PSI for the respective exon is plotted in gray at each amino acid position for reference. **C**, S:N vs PSI for each variant in CM and ES cohorts. **D** and **E**, Bar graphs comparing the S:N of CM and ES variants to gnomAD variants and comparing PSI of CM, gnomAD, and ES variants. * $P < 0.05$.

Application of Pathogenicity Scoring to Determine Likelihood of Incidental Variant Pathogenicity

To determine the pathogenicity of incidentally identified TTNts in the clinical ES referral cohort, we reclassified these variants according to the 2015 ACMG pathogenicity guidelines with incorporation of localization of the variant to areas of high S:N within the criteria.² Initial classification estimated that 78% of the incidentally found TTNts in the ES clinical cohort were pathogenic. Reassignment according to the ACMG guidelines with S:N analysis showed a reduction in proportion that were pathogenic, however, the majority of variants were still deemed pathogenic or likely pathogenic (41% pathogenic, 18% likely pathogenic, and 41% VUS; Figure 4A and 4B). After reassignment, the per position average S:N and the PSI were $4.4 \pm 1.0\%$

and $96.2 \pm 2.4\%$, respectively, for variants reassigned as LP/P and $2.0 \pm 0.4\%$ and $18.5 \pm 4.7\%$ for TTNts reassigned as VUS (Figure 4E and 4F). Overall, these findings suggest an enrichment of variants with higher S:N and PSI among the pathogenic and likely pathogenic incidentally identified variants (Figure 4D).

Validation of Pathogenicity Prediction

To validate the predicted pathogenicity assigned above, we retrospectively reviewed the records of individuals with incidentally found TTNts in the clinical ES cohort who were seen at Texas Children's Hospital. In total, out of 7938 clinical ES referrals, 12 patients met inclusion criteria for review of records and were evaluated at Texas Children's Hospital by cardiology, 7 of which had an echocardiogram (Figure 5), thus the final Texas Children's Hospital clinical validation cohort

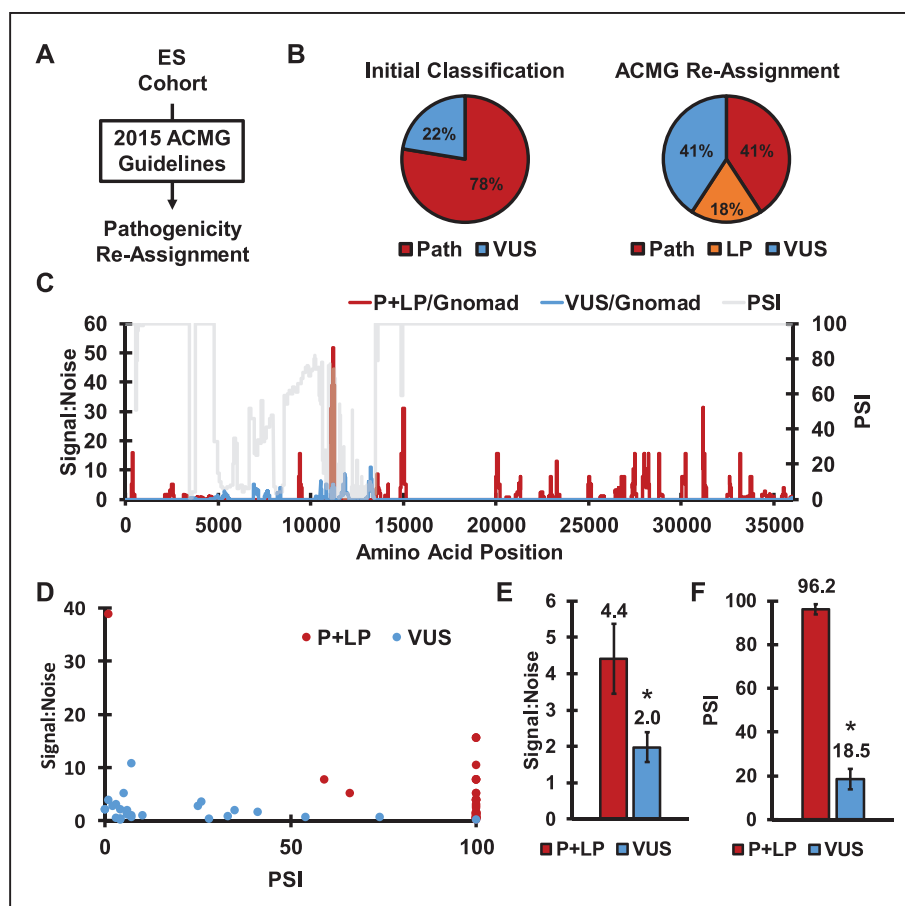


Figure 4. Exome sequencing (ES) variants meeting pathogenic American College of Medical Genetics and Genomics (ACMG) criteria are high percent-spliced in (PSI) and have high signal-to-noise (S:N).

A, Analysis schematic of ES cohort variants reclassified according to 2015 ACMG Pathogenicity Guidelines. **B**, Pie charts demonstrating proportion of pathogenic (Path, red), likely pathogenic (LP, orange), and variants of unknown significance (VUS, blue) before and after reassignment. **C**, Rolling average of the S:N of the nearest 200 variants of the ES cohort compared with genome aggregation database (gnomAD) database by LP/P (red) and VUS (blue) ACMG criteria after reassignment with PSI labeled in gray. **D**, S:N graphed vs PSI of the exon of the variant for each of the ES cohort variants sorted by ACMG criteria. **E** and **F**, Bar graphs comparing relative frequency and PSI of LP/P and VUS variants. * $P < 0.05$.

included 7 patients. The majority (86%) of this cohort was male. The mean age at diagnosis of cardiomyopathy (if present) was 7.4 ± 3.3 years, and the mean length of follow-up was 5.5 ± 2.5 years. Among these 7 individuals, 7 unique TTNtv were identified, 3 (43%) of which were classified as LP/P and 4 (57%) of which were classified as VUS following reassessment of pathogenicity incorporating ACMG criteria with S:N analysis. In those patients with TTNtv classified as LP/P, 67% showed clinical evidence of cardiomyopathy, whereas in patients with TTNtv classified as VUS, 25% showed clinical evidence of cardiomyopathy. This included diagnosis of DCM, left ventricular non-compaction cardiomyopathy, and limb-girdle muscular dystrophy with mildly depressed ventricular function. These findings suggest that, even among this limited cohort, S:N incorporation into ACMG criteria may have a diagnostic role.

DISCUSSION

Pediatric cardiomyopathies are rare, with an estimated annual incidence of 1.1 to 1.5 per 100 000, however, these diagnoses can carry significant morbidity and mortality. Forty percent of pediatric patients with DCM, the most common cardiomyopathy in children, will die or undergo heart transplantation within 2 years of presentation.⁶ Five-year transplant-free survival rates in hypertrophic, restrictive, and left ventricular noncompaction cardiomyopathies are 90%, 30%, and 60%, respectively.¹⁴ In conjunction with the ACMG, the Heart Failure Society of America recommends genetic testing for all pediatric patients with cardiomyopathy.¹⁵ However, TTN is not a gene for which the ACMG recommends reporting of incidental variants, although other more rare variants associated with cardiomyopathy are reportable.¹⁶ In contrast, the recent Babyseq project, which proposed a list of gene loci associated with clinically actionable,

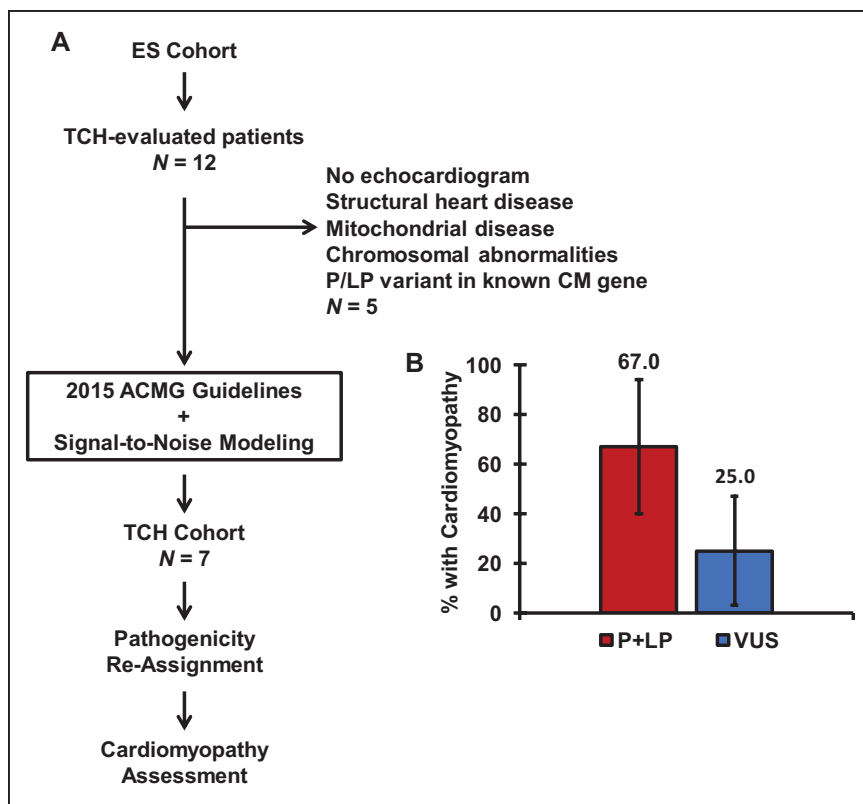


Figure 5. Exome sequencing (ES) cohort evaluated clinically shows trend towards enrichment for cardiomyopathy in patients with variants classified as pathogenic using signal-to-noise (S:N) modified American College of Medical Genetics and Genomics (ACMG) criteria.

A, ES cohort was assigned pathogenicity based on 2015 ACMG criteria. A chart review was then performed on those patients seen at Texas Children's Hospital (TCH) following exclusion of subjects with structural heart disease, mitochondrial disease, or chromosomal abnormalities, or those without echocardiogram.

B, A bar graph of the percentage of variants classified as pathogenic or likely pathogenic (LP/P, N=3) with cardiomyopathy and comparing them to those variants classified as variants of uncertain significance (VUS, N=4).

and, therefore, reportable, variants, did include *TTN*.¹⁷ This highlights that with better ability to distinguish between LP/P and benign TTNts, it would be beneficial to include these variants on the ACMG reportable list, where LP/P are recommended to be reported to physicians as clinically relevant and VUSs are not. In lieu of *TTN*s inclusion on the ACMG-59, physicians can still find themselves needing to interpret the clinical significance of TTNts, as they can be found in instances such as gene panel testing ordered for inappropriate indications and as direct to consumer third party sequencing companies expand, may be reported through these as well. Interpretation of clinical significance of TTNts will, therefore, remain an important part of clinical practice as genetic testing continues to become an expanding modality of clinical assessment.

This is particularly important given the prevalence of *TTN* variants in the general population. In the current study, we found that 63% of the clinical ES referral cohort hosted at least one *TTN* variant, and 1.6% hosted a TTNt. The prevalence of TTNts in the gnomAD cohort was 1.6%. In previous studies, it has been found that the prevalence is \approx 3% in the general population, and a recent meta-analysis found *TTN* variants of any type in up to 23% of patients with DCM.^{8,18} Taken together, it is clear that genetic sequencing has diagnostic value,^{19,20} however, it also introduces a large number of incidentally identified rare variants with unclear diagnostic value. This can present a dilemma for the interpreting clinician as well as possibly undue

distress for families, with the occurrences of these situations only increasing as more patients undergo broad genetic testing and more genes become recommended as reportable. We hypothesize that the solution to this dilemma lies in a more robust role for S:N analysis to help provide clarity in variant pathogenicity.

Previous analyses of *TTN* variants in cardiomyopathy found that variant location can help evaluate for pathogenicity, with the majority of TTNts associated with cardiomyopathy localizing to the A-band coding region.^{9,21} We also found that pathological TTNts were disproportionately localized to the A-band region compared with TTNts found in the gnomAD and clinical ES referral cohorts. The A-band of *TTN* is thought to have many roles including acting as a binding/interacting site for several key proteins, including myosin and myosin binding protein C, thus controlling assembly and length of the thick filament. The A-band region of *TTN* also plays an important role in biomechanical sensing and signaling and contains the *TTN* kinase domain, which is thought to play a role in embryonic sarcomere development and structure maintenance as well as acting as a mechanical sensor to regulate muscle protein expression.^{22–24} In mouse studies, TTNts seem to have little effect on cardiac morphology or function, however, when exposed to a pharmacological or hemodynamic stress, those hearts with a TTNt will dilate and develop systolic dysfunction accompanied by fibrotic changes.²⁵ In studies of cardiomyocytes derived from human induced pluripotent stem cells, TTNts caused baseline impairment of contractility

and impaired cardiomyocyte response to stress.²⁶ Importantly, however, by using an amino acid-level approach, our work demonstrates that not all of the A-band is uniformly associated with disease variant regions. Thus, amino acid-level resolution allows for identification of discrete regions of the A-band that are significantly more pathogenic than others, which can inform which variants are more likely to be clinically impactful.

While knowledge of *TTN* variant coding region can help predict the likelihood of variant pathogenicity, more refined tools are needed, as studies have also found that, while not as common, variants located in the I-band and Z-disk and can also be associated with cardiomyopathy.^{21,27} As evidenced in our analysis, amino acid-level S:N calculations may help inform the diagnostic weight of incidentally identified variants; however, additional studies are needed to validate our S:N variant interpretation in *TTN* and to validate this method more broadly in cardiomyopathy-associated genes, in general. Indeed, replication of this finding in prospectively followed, large, independent cohorts is needed before widespread clinical adoption. We found that not only do LP/P TTNts localize to differing regions of *TTN* compared with benign variants but they are also significantly more likely to localize to regions with higher PSI.

One limitation of the current study is that *TTN*-associated cardiomyopathy may present later in life. Thus, we cannot exclude the possibility of adult-onset disease. However, a recent, although small, study of pediatric patients with cardiomyopathy found an age of presentation at 9 to 18 years of age for those with pathogenic *TTN* variants.³ Thus, among those in the clinical validation cohort with TTNv VUS and normal echocardiogram findings, we may have captured patients before disease onset. Larger studies investigating age of onset of *TTN*-related cardiomyopathy are needed. An additional limitation is the small size of the clinical validation cohort. While we were able to demonstrate the utility of S:N analysis in distinguishing VUS variants from LP/P variants based on comparisons between the ES, gnomAD, and cardiomyopathy cohorts, a larger clinical validation cohort could strengthen this conclusion. To address this limitation, we compared the variants in the ES cohort that were classified as LP/P after S:N analysis to variants found in 2 recent cohort studies that identified TTNts associated with higher risk of clinical cardiomyopathy.^{28,29} We were able to validate that ~8% of the TTNts that we deemed LP/P were also found in the larger cohort study that included a general clinical population and a referral population.²⁹ Given the rarity of each TTNv variant, future studies are needed to further validate their pathogenicity. Larger studies are also needed to determine whether the makeup of TTNts (ie, frameshift, splice-site, and stop gained variants) differ between TTNts found among cardiomyopathy cases and those found in general population cohorts. While the current study determined that

the proportion of variant type differed between the cardiomyopathy, gnomAD, and ES cohorts, relatively small variant numbers precluded direct comparison of the percentage of each variant type individually.

CONCLUSIONS

Incidentally identified TTNts are relatively common in the general population as well as in children undergoing ES. Based on amino acid-level S:N analysis, variants found in the clinical ES cohort were similar to background variants found in the general population, whereas variants found in cardiomyopathy cases had clearly different localization patterns. The majority of *TTN* VUS found in the ES cohort were not associated with signs of cardiomyopathy, and even the pathogenic and likely pathogenic variants in the cohort were not uniformly associated with signs of cardiomyopathy.

ARTICLE INFORMATION

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Affiliations

Department of Pediatrics, Section of Pediatric Cardiology (P.S.C., B.M.S., J.J.L., H.T., H.D.A., S.W.D., J.J.K.) and Department of Molecular and Human Genetics (J.A.R., P.L.), Baylor College of Medicine, Houston, TX. Division of Cardiology, Department of Pediatrics (A.M.B., B.M.S., E.J.P., A.P.L.) and Department of Cell Biology (A.P.L.), Duke University School of Medicine, Durham, NC. Baylor Genetics Laboratories, Houston, TX (J.A.R., P.L.).

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