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The burden of *TTN* variants in the genomic era: analysis of 18,462 individuals from the Solve-RD consortium and general recommendations

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Abstract

Purpose: Titin, the largest protein in the human body, has been associated with several disease phenotypes caused by variants in the *TTN* gene. With around 20% of the population carrying a rare *TTN* variant and over 60 million genomes expected to have been sequenced worldwide by 2025, interpreting these findings presents major challenges. This study analyzed *TTN* variants in the Solve-RD cohort, the European network for unsolved rare disease cases.

Methods: We collected data from 11,072 individuals with suspected rare diseases and 7,390 healthy relatives from the Solve-RD consortium, checking and manually reviewing *TTN* variants. We then used a filtering approach focused on clinical relevance, and we provided updated recommendations based on recent literature.

Results: Among the cohort, 240 individuals (1.3%) carried at least one heterozygous *TTN* truncating variant (TTNtv), with a 3.8% prevalence in the neuromuscular subgroup, primarily composed of unsolved cases. Four individuals received a titinopathy diagnosis. Additionally, 99 participants (0.5%) had a TTNtv in a high-cardiac-PSI exon (>80%), and four had an overt cardiomyopathy.

Conclusion: This study highlights the need for standardized approach to *TTN* variants, and investigation of missing heritability in myopathic individuals with het TTNtv. Establishing consensus on PSI-based thresholds will be essential for assessing cardiac risk and guiding the management of asymptomatic individuals.

Keywords: TTN; titinopathies; secondary findings; cardiomyopathies; neuromuscular disorders

Introduction

In the NGS era, geneticists and clinicians are often faced with results of high complexity and dubious clinical implications.[1] More than 60 million people are expected to have their genome sequenced by 2025 and the total amount of genome sequencing data produced is doubling approximately every seven months.[2,3] As a result, effective approaches are required for handling large volumes of data and for translating them properly into health care management.[4] In genetic reporting, every single variant may raise ethical and clinical issues and have implications that need to be addressed in genetic counselling, both for the proband and their family.[5]

Counselling can be extremely challenging when interpreting titin variants. Titin is the largest known protein in humans and the third most abundant in muscle tissue.[6] The titin (*TTN*) gene (HGNC:12403 ; OMIM #188840) contains 364 exons (363 coding exons and a 5' non-coding exon), including a repeated region with a high degree of complexity and isoform-specific elements. *TTN* transcripts undergo extensive alternative splicing, resulting in several isoforms being differentially expressed in skeletal and cardiac muscles.[7]

In 2002, the first *TTN* pathogenic variant causing a human skeletal muscle disease, the adult-onset tibial muscular dystrophy (TMD or Udd myopathy), was reported.[8] In the last twenty years, the advancement in sequencing technologies has increasingly made it possible to identify clinically relevant variants in this complex gene. Overall, it has been estimated that approximately 15-25% of all cases of non-ischemic dilated cardiomyopathy (DCM) are associated with heterozygous *TTN* truncating variants (TTNtv) making *TTN* one of the most studied genes in cardiology.[9,10] The advances in high throughput sequencing have also led to the identification of many individuals with different forms of skeletal muscle titinopathies, with or without cardiac involvement. With few exceptions, they showed an autosomal

recessive pattern of inheritance (Table 1).[11,12] However, the full landscape of titinopathies is still missing essential pieces, one of the reasons being that *TTN* is probably under-considered both in clinical and diagnostic process, not least because the pathogenicity of missense variants is difficult to prove.[13] Furthermore, many challenges arise with respect to including *TTN* variants in lab reports and whether they should be communicated and discussed. Some of the most relevant technical and interpretative problems are as follows:

- Up to 1% of the population carries a heterozygous TTNtv, and up to 16-20% carries one of many very rare missense variants.[13,14] Thus, many of these are private benign variants or rare variants of uncertain significance (VUS);

- The clinical spectrum of titinopathies is extremely wide, spanning prenatal and neonatal severe forms to mild late adult-onset forms.[12,15–17] Remarkably, DCM due to *TTN* variants shows incomplete penetrance in adulthood and variable severity, suggesting frequent involvement of secondary factors in the affected individuals.[18,19] Consequently, in complex cases or in non-specific phenotypes, *TTN* variants may not be properly addressed because of their difficult interpretation, particularly when access to second-tier analyses is unavailable;

- Finally, pathogenic variants in *TTN* associated with an increased risk of DCM may come as secondary findings in individuals with no family history of cardiac diseases, thus raising ethical issues in defining the actual risk. The inclusion of *TTN* in the ACMG Recommendations for Reporting of Secondary Findings has been particularly challenging and burdensome for clinical labs, given that no specific PSI threshold was provided for 'constitutive exons'.[20] Other studies have proposed or applied a PSI threshold of 90%.[19,21] The same ethical issues arise for adult-onset dominant

titinopathies such as TMD or Hereditary Myopathy with Early Respiratory Failure (HMERF), in particular when found in young people.[22,23]

Here, we describe the approach used for clinical and genetic data interpretation while reanalysing *TTN* genetic findings from 18,462 individuals, including 11,072 participants with a suspected genetic disease, from Solve-RD consortium.[24] To address the aforementioned issues on reporting of *TTN* variants, a multidisciplinary approach integrating genetic analysis with clinical assessment, pathology, imaging, transcriptomic, and proteomic data would represent the best practice. However, this comprehensive approach is unfeasible in most genetic centers, thereby prompting more flexible workflows for interpreting *TTN* variants based on individuals' health status and family history.

Materials and methods

Cohort description

This study includes clinical and genomic data from 18,462 individuals (11,072 affected individuals and 7,390 healthy relatives) submitted to the RD-Connect GPAP as part of the Solve-RD project (http://solve-rd.eu/) by four main European Reference networks (ERNs): European Reference Networks for Rare Neurological Diseases (ERN-RND), Neuromuscular Diseases (ERN-EURO-NMD), Intellectual Disability and Congenital Malformations (ERN-ITHACA) and Genetic Tumor Risk Syndromes (ERN-GENTURIS) (Table 2). Two independent cohorts were collected: the cohort of the 2020 Solve-RD "freeze" (8,068 individuals), and an additional cohort of the 2023 Solve-RD "freeze" (10,394 individuals). Participants from other Undiagnosed Disease Programs have also been included in the 2023 freeze of the Solve-RD cohort (UDN Spain, ERN-PaedCan, ERKNet, ERN-GUARD-HEART, ERN-EYE).

Clinical information and sequencing data were collected and processed as described in Matalonga and others, including deep phenotyping of affected individuals using the Human Phenotype Ontology.[25] Informed consent was obtained for all individuals included in this study. This study adheres to the principles set out in the Declaration of Helsinki.

Variant analysis

For the aim of the current analysis, genomic data from the 18,462 individuals were interrogated for TTNtv (including splicing variants) and missense variants in exons 344 and 364. To exclude variants that did not meet the quality control standards, a manual inspection was conducted with Integrative Genomics Viewer software, and only TTNtv with a minimum variant allele frequency (VAF) of 35% were considered for further examination. This cut-off was chosen to minimize possible false positive variants located in the homologous *TTN*

triplicate region. Variants located in the repeated region have been marked with an asterisk in Supplementary Table 2, to clarify that mapping may not be completely accurate. Overall, there was a minimum read depth DP=8 and a genotype quality GQ=20. Bidirectional coverage was manually checked and variants that did not meet the quality standards were excluded.

For putative splice-affecting variants, only variants in canonical sites with a SpliceAI prediction above 0.6 were included in the analysis.[26] Furthermore, we analyzed non-canonical splice region variants (±8 nucleotides), identifying a total of 33 unique variants in 84 individuals. None of these variants met the ACMG criteria for classification as Likely Pathogenic/Pathogenic, and were not included in the total count of truncating variants.

Only missense variants in exons 344 and 364 were included since pathogenic missense variants in these exons may respectively cause HMERF and TMD.[17,23] Missense variants in other exons were excluded from the analysis, as only a few disease-causing missense variants have been published, and in most cases with insufficient pathogenicity evidence.[27,28]

Additionally, copy number variant (CNV) analysis was performed on individuals with TTNtv.[29] CNVs were filtered to 5% allele frequency and log-likelihood quality score of 30. Five CNVs were examined manually and were all considered false positives due to insufficient quality for accurate CNV calling.

All the identified TTNtv variants with MAF < 1% were subsequently assessed with VarSome Clinical software for ACMG classification. Classification and ACMG criteria applied are shown in Supplementary Table 1. We used the VarSome Clinical software for variant classification, manually reviewing the applied criteria. When the predictions from VarSome and Franklin were conflicting, Franklin's results were also reported. In the case of variants in exon 48 or predicted to affect exon 48 expression (intron 47), the PVS1 criterion was

manually deactivated, following the ClinGen Sequence Variant Interpretation (SVI) Workgroup's guidelines, as, to current knowledge, the expression of this exon is extremely low in both cardiac and muscle tissues (Supplementary Table 1 and 2).[30] Variants with a minor allele frequency (MAF) above the thresholds defined by the ACMG criteria BA1 or BS1 were excluded from the analysis.

In the absence of a specific consensus, we filtered variants according to different cardiac PSI thresholds (Supplementary Figure 2), and eventually considered "cardiac constitutive" those exons with a proportion spliced in cardiac transcripts > 80% (PSI, an estimate of the percentage of *TTN* transcripts that incorporate a particular exon based on RNAseq data) according to Cardiodb (https://www.cardiodb.org/titin/titin_transcripts.php).[31]

Comparison of TTNtv prevalence between cohorts

We assessed differences in TTNtv prevalence (proportion of heterozygotes for pathogenic variants) among cohorts of affected individuals using Chi-squared proportion test. We performed separate analyses for high-cardiac-PSI and low-cardiac-PSI TTNtv. Comparisons were conducted both between individual cohorts and between the combined dataset and the NMD cohort (Table 2 and Supplementary Table 5).

Results

TTN truncating variants (TTNtv) analysis in the Solve-RD cohort

In the cohort of 18,462 individuals, we found 240 (1.3%) individuals heterozygous for TTNtv (Table 2). All the variants were Likely Pathogenic or Pathogenic according to manually revised ACMG criteria, except variants in intron 47 and exon 48, which were reclassified as Variants of Uncertain Significance.

Of these, 168 are probands suspected of having a genetic disease (1.5% of the patients) and 72 are healthy relatives (1.0% of the healthy relatives). Of the 168 affected individuals heterozygous for a TTNtv, 35 (21%) have been classified as "solved" to date, but only 4 of them have a titinopathy diagnosis (Supplementary Table 1).

Cohort overview

The cohort has an average age at last examination that falls within the range of young adulthood. Even though collection of age data is beyond the scope of the Solve-RD project, we managed to collect data for a subset of individuals (158 individuals), with a mean age of 33 years. Among the 99 individuals carrying a heterozygous TTNtv in a high-cardiac PSI exon (>80%), we retrieved age data for 67 individuals. The age range is 4–79 years (average=32 years and standard deviation=21). Of these 67, 11 are healthy relatives, with an age ranging between 30 and 56 (average=44 years). The cohort predominantly consists of individuals of non-Finnish European origin, reflecting the geographic distribution of the main contributing countries.

Biallelic *TTN* variants leading to titinopathy diagnosis

We found 3 participants carrying biallelic TTNtv with phenotypes compatible with biallelic titinopathies, two belonging to ERN-EURO-NMD and one to ERN-RND, all presenting with

muscular hypotonia, congenital muscular dystrophy, and two also with contractures (Supplementary Table 1). The age at last examination was 8, 11, and 13 years, respectively. Each of these participants carries a variant in a cardiac constitutive exon, but no overt cardiomyopathy was observed. Another individual carries a known pathogenic missense variant in exon 364, in compound heterozygosity with a TTNtv in exon 327, and presented with a limb-girdle phenotype compatible with LGMDR10 (Table 1).

TTNtv prevalence analysis by cohort

Overall, 237 individuals carrying a heterozygous TTNtv were identified: 157 are unique variants, including 115 truncating variants, and 42 splicing variants (Supplementary Table 1).

A significant enrichment of TTNtv is observed in the ERN-EURO-NMD cohort (Table 2). Forty-one out of 1,067 (3.8%) affected individuals from ERN-EURO-NMD carry a heterozygous TTNtv. Additionally, seven unaffected relatives (1.8%) also carry the variant. Among other cohorts, the prevalence of TTNtv was in the range 1.0-1.8%.

TTNtv in cardiac constitutive exons

Of the 237 individuals carrying a heterozygous TTNtv, four affected participants showed an overt cardiomyopathy. Three out of four cardiomyopathic individuals were between 53 and 56 years old at the last evaluation. One is an undiagnosed myopathy patient with a history of neonatal hypertrophic cardiomyopathy, resolved initially, and a borderline concentric left ventricular hypertrophy at mid ventricular level, and mildly dysplastic mitral valve with mild regurgitation noted at age of 15 years. Two of the individuals aged approximately 50 years have a TTNtv considered “medically actionable”, being in the A-band, distal I-band, or in exon 49, which are regions with a strong association with causal DCM variants and a cardiac PSI of approximately 100%. One has a complex phenotype resembling oculopharyngodistal myopathy, and the other has “polycystic kidney and liver dysplasia” “ischemic stroke”,

"carotid artery aneurysm" among other features; they are both still considered unsolved. The other two participants with clinically overt cardiac involvement carry TTNtv located in exons less commonly associated with DCM. One carries a splicing variant (NM_001267550.2: c.30683-1G>T; NC_000002.11:g.179563642C>A) predicted to cause the splice acceptor site loss (SpliceAI score: 0.99) resulting in premature termination. To date, exon 113 has never been associated with DCM and has a PSI value of 80%. The same variant has also been identified in 5 other individuals, none of whom had reported signs of cardiomyopathy. A young myopathic individual has a splicing variant (NM_001267550.2: c.4645+2T>C; NC_000002.11:g.179642145A>G) predicted to cause splice donor site loss (SpliceAI score: 0.68) and a 114 bp deletion of exon 26, constitutively expressed in the heart.

We then assessed how many individuals carry TTNtv in a high-cardiac-PSI exon in the rest of the cohort. Out of 157 distinct TTNtv variants, we filtered out unique variants in exons with a PSI lower than 80% in heart (Figure 1). Among all, eighty unique variants carried by 99 individuals (42% of total individuals heterozygous for a TTNtv, 0.5% of the Solve-RD cohort), are located in an exon with cardiac PSI > 80%.[19] Sixty unique variants carried by 69 individuals are located in the A-band, distal I-band, or in exon 49.[32] Twenty unique variants carried by 30 individuals are located in other regions of titin. Overall, of the 99 individuals carrying a TTNtv in a high-cardiac-PSI exon, 44 were recruited from ERN-ITHACA, 30 from ERN-EURO-NMD, 15 from ERN-RND, six from ERN-EpiCARE, four from ERN-GENTURIS. This distribution aligns with the proportions of individuals recruited from each ERN, except for the ERN-EURO-NMD cohort, which, despite representing only 8% of the Solve-RD cohort, accounts for 30% of individuals with a TTNtv in high-cardiac-PSI exons. Out of 99, twelve participants are now classified as "solved" with non-titinopathy diagnoses. In 4 individuals, cardiac involvement was excluded prior to conducting the genetic test.

Non-truncating variants in exon 344 and 364

Forty-eight individuals (31 affected participants and 17 unaffected relatives) carry missense variants in exon 344, for a total of 14 unique variants: three of them are classified as Likely Benign and eleven as VUS (Supplementary Table 3). Two of them presented "recurrent respiratory infections" and one "neonatal respiratory distress", while others presented some muscle weakness and fatigability, but no one showed signs and symptoms considered specific for HMERF. Two participants have to date been classified as "solved" for reasons other than titin.

Thirty-eight individuals (21 affected probands and 17 unaffected relatives) carry rare missense variants in the last titin exon 364. Overall, there were nine unique variants, six of them classified as Likely Benign, two as VUS, and one as Pathogenic (Supplementary Table 4). The pathogenic variant (NM_001267550.2: c.104840T>C p.(Ile35944Asn); NC_000012.12:g.57102340T>C) was found in three myopathic participants, belonging to the same family (FAM015). Although they had been considered diagnosed, only one of them carries a TTNtv on the other allele, while the phenotype in the other relatives seems not to be compatible with the mild clinical course of a TMD, thus suggesting the presence of other elusive variant/s.

Discussion

In the NGS-era, the secondary finding of high-cardiac-PSI TTNtv could lead to an increasing demand for genetic counselling, potentially involving approximately 0.5-1% of the population.[14] We may estimate that, among the 60 million genomes expected by 2025 worldwide, about 300.000 may have actionable *TTN* variants.[33] Thereby, a good understanding of titin-related diseases will be of crucial importance for proper variant interpretation and appropriate counselling. We consider our cohort as a valuable model for assessing the prevalence of *TTN* variants and discussing the complex issues of interpretation and counselling raised by the wide titinopathies spectrum.

TTNtv and cardiomyopathy

Approximately 13% of all DCM cases are linked to TTNtv, and the estimated prevalence of DCM in the adult general population is roughly 1 in 250.[32] Based on these data, the estimated prevalence of *TTN*-associated DCM would be 1 in 10,000 (0.052%). With a 0.5% prevalence of high-cardiac-PSI TTNtv in our cohort, expected penetrance would be 0.00052 / 0.005 = 10.4%, meaning that 10.4% of heterozygous individuals would be expected to develop the disease (95% CI: 7.2% – 14.6%).[9,32] Notably, prevalence of high-cardiac-PSI TTNtv in our cohort, mainly of non-Finnish European origin, is consistent with what was found by Shetty et al. in their work on a large cohort of African and European ancestry.[34] This further confirms that ethnicity/genetic ancestry are not major contributors to disease expression in titinopathies.

To date, almost all the studies have focused on DCM probands or individuals with a family history of DCM, which introduces potential selection bias.[9,18] This bias does not apply to our study, as the individuals were not selected based on a personal or family history of DCM. On the contrary, our study serves as a model for patient cohorts in clinical genetics centers, as

only a very small portion of participants were screened specifically for cardiomyopathy before undergoing genetic analysis. In four individuals with TTNtv, DCM and arrhythmia were ruled out; in other cases, it remains uncertain whether a cardiac evaluation was conducted. On subsequent re-evaluation after genetic testing, clinical or subclinical cardiomyopathy will most likely be discovered in some individuals, but a prospective collection of this data goes beyond the scope of Solve-RD. The age of the individuals at the last assessment is not routinely collected by Solve-RD, but the estimated composition of the cohort (average age for affected individuals 30 years, 44 years for unaffected relatives) can be considered in line with a clinical genetics setting, including predominantly children, adolescents and young adults. Our data on DCM "pre-test" penetrance in young individuals carrying a heterozygous high-cardiac-PSI TTNtv align with the current literature.[18,35] However, as expected, it remains lower when compared to studies on large cohorts of cardiology and myopathy patients.[36,37]

Defining which TTNtv variants should be considered “actionable” is complex, as they are associated with a low penetrant disease.[20,32,38] Notably, a variant could be pathogenic for an autosomal recessive titinopathy, but may be uncertain in the context of autosomal dominant DCM and not diagnostic if found in a patient with only cardiomyopathy. For example, current knowledge does not provide a clear threshold of cardiac PSI beyond which a *TTN* variant can be considered detrimental to cardiac function. Although A-band TTNtv variants are the most well-established in their association with DCM, recent literature would suggest that a TTNtv in a high-PSI exon is actionable regardless of *TTN* domain.[19] Indeed, after noting the presence of a participant with DCM carrying a variant in an exon with a PSI of 80%, we evaluated several thresholds to select “high”-cardiac-PSI exons (Supplementary Figure 2). We set a threshold of 80% so that we can include all the participants with an overt cardiomyopathy without a dramatic increase in the number of individuals with a variant in a high-PSI exon

(from 38% to 40% of all the participants with a heterozygous TTNtv). Of course, this was an arbitrary choice, since we are aware that our cohort has an average age that doesn't allow us to fully appreciate the penetrance. A large study that would evaluate specificity and sensitivity of different cardiac PSI thresholds would be needed, based also on cohorts with older average age.[39]

Current guidelines recommend that TTNtv in cardiac constitutive exons be reported regardless of the clinical indication, as they increase the risk of developing DCM, if the informed consent for secondary findings has been accorded.[40] This recommendation includes splicing variants affecting cardiac constitutive exons with high splicing prediction scores, some of which may be in-frame but could skip exons that are crucial for the protein.[41,42] Overall, *TTN* variants should be reported in a genetic lab report using the criteria summarized in Supplementary Figure 1, making sure to use the MANE isoform (NM_001264550.1) including meta-transcript only exons. Some useful tools to look at the different canonical titin isoforms and to convert variants from one isoform to another may be found in Table 3. When considering all TTNtv in exons with an adult cardiac PSI > 80%, the penetrance in our cohort is 4%; none of them has other family members carrying the same TTNtv included in the study. In the literature, the median age of DCM onset in individuals heterozygous for TTNtv is approximately 50 years, with some studies reporting DCM penetrance increasing from 34% at 40 years to 93% at 80 years.[36] However, it is now clear that, as indicated by our results, TTNtv identified during sequencing for other indications, cannot be assumed to confer the same disease risk as those identified in the context of disease and are less likely to be clinically actionable.[32] For reasons not fully understood, the penetrance is also typically higher for male individuals, and this should be taken into consideration during counselling.[34] Although some environmental risk factors are known, further studies are needed to determine additional modifier factors and other multilocus genetic determinants to account for the risk

of DCM.[35] On the other hand, several studies have highlighted the importance of genetic screening in cohorts of DCM patients, as it allows for a personalized approach in both therapy and cardiac device installation. There are no guidelines in the literature regarding follow-up for individuals without a cardiac phenotype and without a family history of cardiomyopathy. However, the follow-up strategy for these individuals should differ from the current recommendations by the European Society of Cardiology, the American Heart Association, and the American College of Cardiology for family members of an affected proband. For instance, based on the evidence from the literature and this study, we propose that it might follow a less frequent schedule, such as every 5 years or even longer, depending on the individual's age. Clinical and genetic cascade analysis in relatives is suggested when the proband is affected, according to each participant's consent; currently, there is no clear evidence of the benefit of cascade testing in families without a history of DCM. During genetic counselling, risk in offspring must be mentioned with a focused approach on the penetrance and on the risk of recessive titinopathies, considering family history and literature.[16] If the phenotype is suggestive of DCM but the variant has never been reported, we strongly suggest reporting it and/or contacting a consortium (e.g., Cardiovascular Genomics and Precision Medicine Group, www.cardiodb.org).

TTNtv and myopathy

Currently, most of the TTNtv, regardless of their position, are thought to only cause recessive muscle diseases.[12] We found that approximately 4% of myopathy cases carry a single TTNtv versus a cumulative frequency of up to 1% in the general population. These findings may suggest that cases of myopathy involving titin are being underdiagnosed, potentially due to overlooked variants (e.g., VUS, variants in non-coding regions, etc) or more complex mechanisms not yet understood. As aforementioned, the identification of a TTNtv in a myopathic individual should be always reported although the genetic report/the specialist

should state clearly that this finding does not necessarily indicate a diagnosis of titinopathy and additional tests are required. The clinical interpretation needs to rely on a careful application of the ACMG/AMP guidelines, and we suggest following the steps in Figure 2.

Even a comprehensive study, however, may not always be conclusive. Unsolved cases could be due to elusive variants in the highly homologous triplicate repeat region where DNA and RNA sequencing may fail to call or map variants. It is likely that techniques providing greater coverage in problematic regions (e.g., long read sequencing) will enable us to overcome specific technical issues and improve variant detection in the triplicate region.[43]

Another limitation of the current study is that it does not consider digenic or more complex mechanisms; for instance, a digenic *TTN-SRPK3* (HGNC:11402) myopathy has been recently described.[44] Large international initiatives will most probably allow the identification of additional examples of digenic and non-Mendelian genetic mechanisms involving *TTN* variants.[45]

The interpretation of missense variants remains challenging. Heterozygous pathogenic missense variants in exon 364 account for late-onset TMD; however, in cases with a more severe phenotype, as in the family reported here (FAM015, Supplementary Table 1 and S4), the presence of an additional variant in trans should be considered. Although few missense variants in other exons with a possible clinical effect have been reported, we still lack a clear consensus on how to assess their pathogenicity, probably underestimating their impact.[28]

The prevalence of rare missense *TTN* variants is higher than TTNtv (16-20% approximately), but few of them have been associated with an increased risk of cardiomyopathy or have been proven to cause recessive titinopathies.[46] As for the current guidelines, neither TMD variants nor HMERF associated variants would be reported as secondary findings. Instead, if a missense pathogenic variant in exon 364 is found in a myopathic individual with a phenotype

compatible with recessive titinopathy, additional data review for a second variant should be performed.

Preconception counselling for individuals heterozygous for *TTN* pathogenic variants

Our main recommendations for preconception counselling for individuals heterozygous for a TTNtv are summarized in Supplementary Figure 3. If both partners carry TTNtv, the couple may either incur recurrent miscarriages or have a child with a congenital myopathy (if one of the mutated alleles still results in an 'almost-full length protein').[15] The only exception is the presence of a TTNtv in the last five exons that would result in a childhood or later onset myopathy.[12]

TTN is already included in some reproductive genetic carrier screenings, offered both by public and private institutions (https://www.mackenziesmission.org.au/what-conditions-are-screened/). Overall, if an individual has a TTNtv, then the partner may be offered a carrier screening. Prenatal genetic analysis should be offered if the couples request it, and, also, pre-implantation genetic testing (PGT) after in vitro fertilization (IVF) can be an option.[47] Given the complexity and sensitivity of these cases, we recommend contacting a consortium or a specialized center for a proper evaluation of the clinical meaning of the identified TTNtv.

Limitations and conclusion

This study has some limitations. First, due to the data collection method and the purpose of the Solve-RD project, we were able to retrieve the age at the last evaluation for only about 67% of the individuals, and to provide only an estimate of the cohort average age (33 years). Secondly, not all individuals underwent a cardiological evaluation prior to genetic testing. As a result, the reported cardiomyopathy cases include only those who were overtly symptomatic before testing, as is often the case in genetic centers. Indeed, this study was not intended to

focus on DCM penetrance analysis, although we noted that penetrance, as in other studies on young cohorts, seems to be low.

It rather aimed to describe the approach to *TTN* variants as primary and secondary findings in large cohorts of heterogeneous individuals undergoing genetic testing for various reasons. For this purpose, it adopted a broad perspective, including the assessment of reproductive risk. Moreover, with our data we highlighted the lack of a precise cardiac PSI cut-off to define DCM. Also, we proved a statistically significant enrichment of TTNtv variants in the NMD cohort of unsolved cases. This finding underscores the need for international collaboration to uncover the underlying causes of missing heritability in titinopathies.

Data Availability

All variants of clinical interest are available in supplemental tables. More detailed information is available from the corresponding authors upon request. Genetic and phenotypic data for all individuals is accessible to registered users of the RD-Connect GPAP (platform.rd-connect.eu). All raw and processed data files can be made available at the European Genome-Phenome Archive (https://ega-archive.org/ under the Solve-RD study EGAS00001003851), following approval from the Solve-RD Data Access Committee.

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Author Contributions

Conceptualization: M.S., M.F.D.F., M.J.; Data Curation: I.P., L.M.B., G.D., S.N.G., A.T., A.H., S.B., H.G., L.V., R.V.,R.V.,M.G.,S.B.,H.L.,G.L.,K.E.,M.C.,A.P.,A.S.,G.B.,J.A.J.V., Solve-RD consortium; Formal Analysis: M.F.D.F., M.S., I.P., L.M.B., G.D., S.N.G; Investigation: M.F.D.F., M.S.; Methodology: M.F.D.F., M.S., B.U.; Supervision: M.S., B.U.; Validation: I.P., L.M.B.; Visualization: M.F.D.F., M.S., I.P.; Writing-original draft: M.F.D.F.; Writing-review and editing: M.S., B.U., A.T., A.S., S.L., K.E., G.D.

Ethics Declaration

Ethical approval for this study falls under HUS/16896/2022. Ethics committee/IRB of University of Tübingen gave ethical approval for this work. Reference to the respective ClinicalTrials.gov Nr.: NCT03491280 (https://clinicaltrials.gov/study/NCT03491280). Written informed consent for data sharing within Europe for the purpose of research was obtained from all recruited individuals or their parents/legal guardians where appropriate. The responsibility of checking the data is suitable for submission to the RD-Connect GPAP and Solve-RD, including informed consent, lies within the data submitter as required by their Code of Conduct and Data Sharing Policy, respectively. In some cases, individuals had to be re-consented prior to data submission. This study adheres to the principles set out in the Declaration of Helsinki.

Conflicts of interest

The authors declare no conflict of interest.

Supplemental file listing

Supplementary Figure 1. Summary of recommendations for genetic reporting of *TTN* variants.

Supplementary Figure 2. Percentage of individuals heterozygous for a TTNtv in high-cardiac-PSI exons in the Solve-RD cohort, with and without overt DCM, according to different cardiac PSI threshold (100% PSI; >90% PSI; >80% PSI; >40% PSI).

Supplementary Figure 3. Summary of recommendations for preconception counselling in individuals heterozygous for a TTNtv.

Supplementary File 1. Supplementary Table 1: All TTNtv in the Solve-RD cohort, with participants' details; Supplementary Table 2: TTNtv found in heterozygosity in the Solve-RD cohort, with participants' details and ACMG/AMP classification; Supplementary Table 3: *TTN* variants in exon 344 in the Solve-RD cohort, with participants' details; Supplementary Table 4: *TTN* variants in exon 364 in the Solve-RD cohort, with participants' details; Supplementary Table 5: p-values for the Chi-square proportion test performed on the prevalence of TTNtv (total and with different cardiac PSI) in various cohorts compared to the NMD cohort.

Supplementary File 2. Full list of Solve-RD consortium members and associated partners

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Figure legends

Figure 1. Filtering process (Figure 1a) and TTNtv distribution (Figure 1b) in the Solve-RD cohort according to adult cardiac PSI. “High cardiac PSI” > 80% PSI in adult cardiac muscles; “low cardiac PSI” < 80% PSI in adult cardiac muscles.

Figure 2. Recommendations on clinical and diagnostic management of TTNtv, in cases with TTNtv in exons with high cardiac PSI (Figure 1a) and in cases of myopathic patients (Figure 1b)

Table 1. *TTN* allelic disorders and inheritance

Disorder	Phenotype	Transmission
Dilated cardiomyopathy (DCM) (OMIM #604145)	Overt DCM or increased risk of DCM (penetrance is still discussed)	AD
Hypertrophic cardiomyopathy (HCM) *(OMIM #613465)*	*Overt HCM, TTNtv typically in association with pathogenic variants in other cardiac genes*	*AD*
Udd distal myopathy/ tibial muscular dystrophy (TMD) (OMIM #600334)	Distal myopathy with degeneration of tibialis anterior, fourth to seventh decades	AD, variants in exon 364
Hereditary myopathy with early respiratory failure (HMERF) (OMIM #603689)	Respiratory failure and proximal (or initial distal) muscle weakness	AD, variants in exon 344
Autosomal recessive limb-girdle muscular dystrophy (LGMDR10) (OMIM #608804)	Childhood or young adult-onset myopathy, limb-girdle involvement, variable severity	AR
Adult-onset distal titinopathy	Distal myopathy with degeneration of tibialis anterior and soleus	AR, AD (only large inframe CNV)
Adult-onset proximal rimmed vacuolar myopathy	Quadriceps and soleus fatty replacement	AR
Congenital titinopathies	Depending on variants, prenatal phenotypes (reduced fetal movements,	AR, AD (only large inframe CNV)

	arthrogryposis, hypotonia)	
Prenatal lethal titinopathy	Severe muscle hypotrophy, arthrogryposis, possible fetal hydrops, syndromic-like signs	AR (variants in exon with high prenatal PSI)
Digenic titinopathy	Congenital, slowly progressive myopathy, predominantly proximal and axial, affecting the lower more than the upper limbs	Digenic (*TTN, SRPK3*) Predominantly males

Legend: Associations reported in a minority of cases and still under debate are shown in italics.

Table 2. Solve-RD cohort

ERNs	**Affected individuals**				**Unaffected relatives**			
	Total number of individuals	Het for a TTNtv (%)	Het for a TTNtv in a high-cardiac-PSI exon (PSI > 80%) (%)	Het for a TTNtv in a low-cardiac-PSI exon (PSI < 80%) (%) *excluding intron 47, exon 48*	Total number of relatives	Het for a TTNtv (%)	Het for a TTNtv in a high-cardiac-PSI exon (PSI > 80%) (%)	Het for a TTNtv in a low-cardiac-PSI exon (PSI < 80%) (%) *Excluding intron 47, exon 48*
ERN-ITHACA	4287	61 (1.4%)	24 (0.6%)	25 (0.6%)	5967	57 (1.0%)	20 (0.3%)	20 (0.3%)
ERN-RND	3501	42 (1.2%)	11 (0.3%)	15 (0.4%)	417	8 (1.9%)	4 (1.0%)	2 (0.5%)
ERN-EURO-NMD	**1067**	**41 (3.8%)[a]**	**27 (2.5%)[b]**	**11 (1.0%)**	400	7 (1.8%)	3 (0.8%)	4 (1.0%)
ERN-GENTURIS	448	8 (1.8%)	4 (0.9%)	2 (0.4%)	30	0	0	0
ERN-EpiCARE	1400	14 (1.0%)	6 (0.4%)	6 (0.4%)	294	0	0	0
ERN-RITA	206	2 (1.0%)	0	1 (0.5%)	75	0	0	0
UDN-Spain	36	0	0	0	49	0	0	0
ERN-PaedCan	71	0	0	0	130	0	0	0
Not_Applicable	46	0	0	0	3	0	0	0
ERN-GUARD-HEART	5	0	0	0	9	0	0	0
ERKNet	3	0	0	0	10	0	0	0
ERN-EYE	2	0	0		6	0	0	0
Sum	*11072*	*168 (1.5%)*	*72 (0.7%)*	*60 (0.5%)*	*7390*	*72 (1.0%)*	*27 (0.4%)*	*26 (0.4%)*

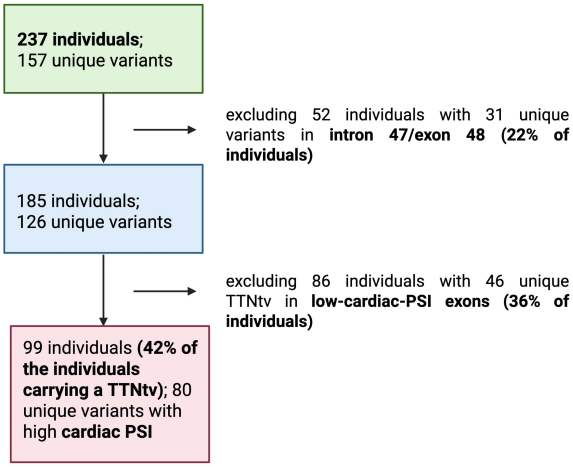
Legend: [a] p-value (ERN-EURO-NMD vs all the other cohorts) = 4.99e-12

[b] p-value (high-cardiac-PSI ERN-EURO-NMD vs all the other cohorts) = 2.98e-08

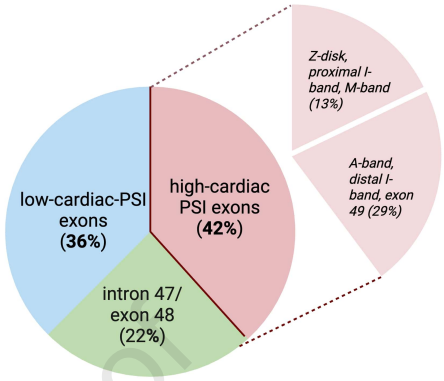
Table 3. Useful tools for *TTN* variants

Useful links and tools	**Aim**
https://www.cardiodb.org/titin/titin_transcripts.php	To check variants position and exons included in the different transcripts
https://mutalyzer.nl/position-converter	To convert variants from one isoform to another one
https://databases.lovd.nl/shared/genes/TTN	To check already reported variants
https://fraternalilab.kcl.ac.uk/TITINdb	To integrate titin structure, sequence, isoforms, variants, and disease information
https://www.uniprot.org/uniprotkb/Q8WZ42	To check the protein domains and three-dimensional structure

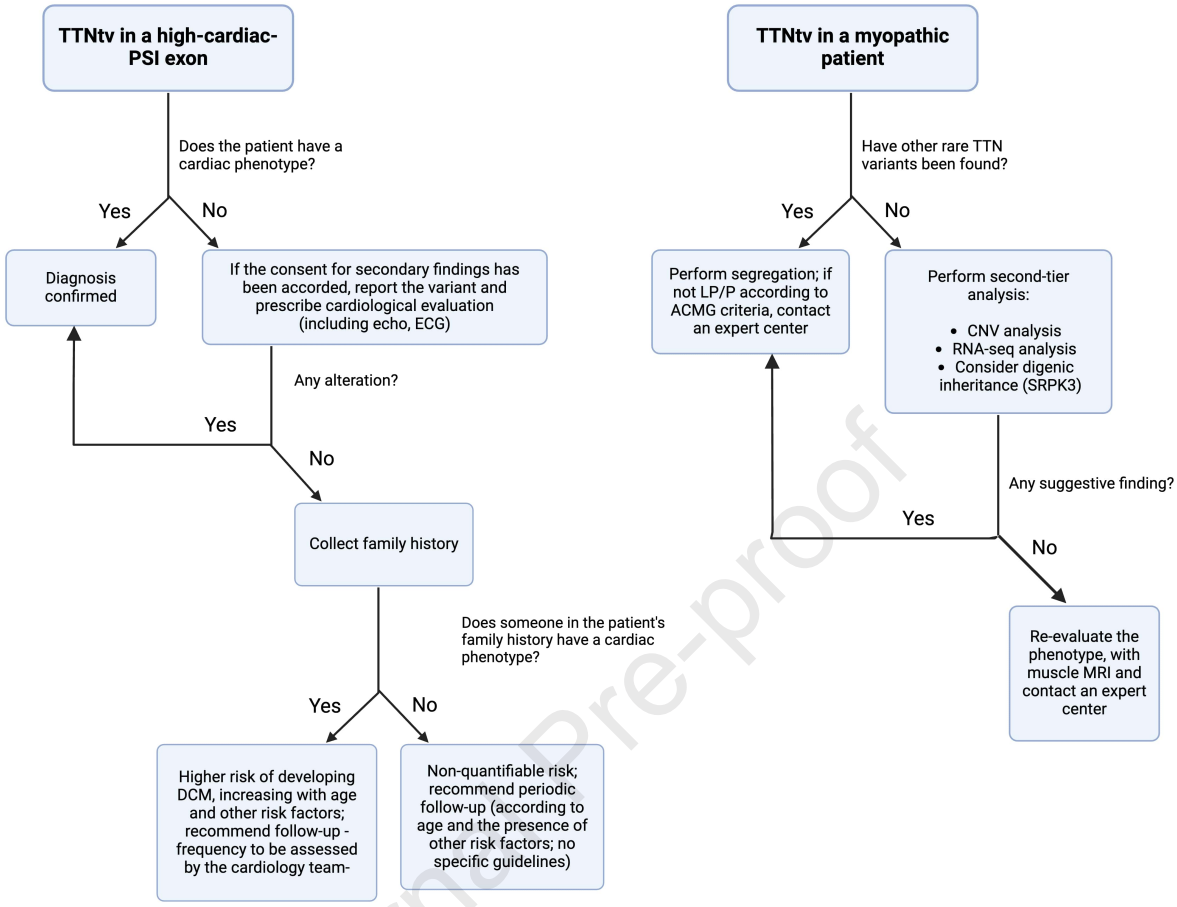
Solve-RD individuals carrying a het TTNtv



Solve-RD individuals carrying a high-cardiac-PSI TTNtv



Distribution of TTNtv variants across TTN regions



Solve-RD consortium

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