Titin cardiomyopathy leads to altered mitochondrial energetics, increased fibrosis and long-termlife-threatening arrhythmias

ESC European Society of Cardiology

European Heart Journal (2018) **39**, 864–873
doi:10.1093/eurheartj/ehx808

CLINICAL RESEARCH
Heart failure/cardiomyopathy

Titin cardiomyopathy leads to altered mitochondrial energetics, increased fibrosis and long-term life-threatening arrhythmias

Job A.J. Verdonschot[1,2], Mark R. Hazebroek[1], Kasper W.J. Derks[1,2], Arantxa Barandiarán Aizpurua[1], Jort J. Merken[1], Ping Wang[2], Jörgen Bierau[2], Arthur van den Wijngaard[2], Simon M. Schalla[1,3], Myrurgia A. Abdul Hamid[4], Marc van Bilsen[1], Vanessa P.M. van Empel[1], Christian Knackstedt[1], Hans-Peter Brunner-La Rocca[1], Han G. Brunner[2,5], Ingrid P.C. Krapels[2], and Stephane R.B. Heymans[1,6,7]*

[1]Department of Cardiology, Maastricht University Medical Centre, Center for Heart Failure Research, Cardiovascular Research Institute Maastricht (CARIM), University Hospital Maastricht, P. Debyelaan 25, 6229 HX Maastricht, The Netherlands; [2]Department of Clinical Genetics and School for Oncology & Developmental Biology (GROW), Maastricht University Medical Center, 6202 AZ Maastricht, The Netherlands; [3]Department of Radiology and Nuclear Medicine, Maastricht University Medical Centre, P. Debeylaan 25, 6229 HX Maastricht, The Netherlands; [4]Department of Pathology, Maastricht University Medical Centre, P. Debeylaan 25, 6229 HX Maastricht, The Netherlands; [5]Department of Human Genetics, Radboud University Medical Center, Donders Institute for Brain, Cognition and Behaviour, Nijmegen, 6500 GA, The Netherlands; [6]Department of Cardiovascular Research, University of Leuven, UZ Herestraat 49, 3000 Leuven, Belgium; and [7]Netherlands Heart Institute (ICIN), Moreelsepark 1, 3511 EP Utrecht, The Netherlands

Received 4 August 2017; revised 13 October 2017; editorial decision 21 December 2017; accepted 22 December 2017; online publish-ahead-of-print 25 January 2018

See page 874 for the editorial comment on this article (doi: 10.1093/eurheartj/ehy109)

Aims Truncating titin variants (TTNtv) are the most prevalent genetic cause of dilated cardiomyopathy (DCM). We aim to study clinical parameters and long-term outcomes related to the TTNtv genotype and determine the related molecular changes at tissue level in TTNtv DCM patients.

Methods and results A total of 303 consecutive and extensively phenotyped DCM patients (including cardiac imaging, Holter monitoring, and endomyocardial biopsy) underwent DNA sequencing of 47 cardiomyopathy-associated genes including *TTN*, yielding 38 TTNtv positive (13%) patients. At long-term follow-up (median of 45 months, up to 12 years), TTNtv DCM patients had increased ventricular arrhythmias compared to other DCM, but a similar survival. Arrhythmias are especially prominent in TTNtv patients with an additional environmental trigger (i.e. virus infection, cardiac inflammation, systemic disease, toxic exposure). Importantly, cardiac mass is reduced in TTNtv patients, despite similar cardiac function and dimensions at cardiac magnetic resonance. These enhanced life-threatening arrhythmias and decreased cardiac mass in TTNtv DCM patients go along with significant cardiac energetic and matrix alterations. All components of the mitochondrial electron transport chain are significantly upregulated in TTNtv hearts at RNA-sequencing. Also, interstitial fibrosis was augmented in TTNtv patients at histological and transcript level.

Conclusion Truncating titin variants lead to pronounced cardiac alterations in mitochondrial function, with increased interstitial fibrosis and reduced hypertrophy. Those structural and metabolic alterations in TTNtv hearts go along with increased ventricular arrhythmias at long-term follow-up, with a similar survival and overall cardiac function.

Keywords Cardiomyopathy • Titin • Genetics • Prognosis • Genotype–phenotype • Mitochondrial

* Corresponding author. Tel: +31 43 3884304, Fax: +31 43 3882952, Email: s.heymans@maastrichtuniversity.nl

Introduction

Dilated cardiomyopathy (DCM) has a prevalence of at least 1:250 and is an important cause of heart failure (HF) and transplantation in young adults.[1,2] The disease is familial in up to 45% of patients. Up to one quarter of DCM patients have a combined genetic and environmental aetiology.[1,3,4] Approximately 15–20% of DCM patients have a truncating *TTN* variant (TTNtv), largely enriched in the titin A-band.[5,6] Furthermore, TTNtv located in constitutive exons (spliced in >90% of transcripts) are strongly associated with DCM, irrespective of their position in *TTN*.[5] As the pathogenicity of these TTNtv becomes increasingly evident, the non-genetic factors determining their penetrance remain largely unknown. Recently, chemotherapy was reported as a potential factor uncovering TTNtv effects.[7] Also, peripartum changes can be a factor uncovering the underlying genetic mutation as TTNtv are prevalent in patients with peripartum cardiomyopathy.[8] However, the overall involvement of non-genetic acquired factors is largely unclear.

Information regarding the clinical phenotypes and outcomes of DCM patients with TTNtv mutations at long-term is also sparse and often contradictory.[5,9] An increase in arrhythmias at early presentation, but a paradoxically more treatable phenotype at follow-up was reported in TTNtv patients.[9,10] However, long-term data on cardiac arrhythmias—and its relation to overall survival—are lacking.

Metabolic changes were reported in the TTNtv hearts of rats as compared to normal hearts.[6] Whether such metabolic changes reflect overall cardiac stress or are directly caused by the *TTN* mutation remains unknown. In ischaemic aetiologies of HF, metabolomics have provided valuable insights on the cardiometabolic disease state with additional prognostic value.[11,12] By contrast, there is a paucity of data in non-ischaemic DCM.[13] Data on cardiac structural and molecular changes in TTNtv patients are missing, but strongly needed to understand the pathophysiology of TTNtv.

Our aim in this study was to better understand and dissect the phenotypic and clinical significance of the TTNtv at long term. We undertook deep phenotyping, longitudinal clinical follow-up and analysis of molecular pathways in endomyocardial biopsies of a large consecutive cohort of patients with TTNtv compared to TTNtv negative DCM patients.

Methods

Study design

All clinical and follow-up data were collected from 2004 until July 2016 in the Maastricht Cardiomyopathy Registry.[3] Pedigree analysis and sequencing of a 47-cardiomyopathy-associated gene panel including *TTN* was performed in 303 consecutive, unrelated cardiomyopathy patients since 2012. The study was performed according to the declaration of Helsinki and was approved by the Medical Ethical Committee of Maastricht University Medical Centre. All patients gave written informed consent. All patients received treatment according to the latest ESC guidelines on acute and chronic HF therapy.[14]

Patient recruitment

Inclusion criteria for the inclusion of patients referred to our centre with unexplained LV dysfunction were: (i) DCM defined as LVEF < 50% with an indexed left ventricular end diastolic diameter (LVEDDI) > 33 mm/m^2 (men) or >32 mm/m^2 (women) measured by echocardiography; or a hypokinetic non-dilated cardiomyopathy (HNDC) defined as LVEF < 50% with an LVEDDI ≤ 33 mm/m^2 (men) or ≤ 32 mm/m^2 (women) measured by echocardiography.[15,16] This mixed population is further referred to DCM in this paper; (ii) sequencing of *TTN*; (iii) age ≥18 years; (iv) written informed consent.

Exclusion criteria for the Maastricht Cardiomyopathy Registry included: (i) previous history of myocardial infarction and/or significant coronary artery disease (stenosis > 50%) using coronary angiography sufficient to explain cardiac dysfunction; (ii) primary valvular disease (mitral regurgitation grade ≥3, aortic regurgitation grade ≥2, or aortic stenosis with AVA < 1 cm^2); (iii) hypertensive heart disease; (iv) congenital heart disease; (v) acute myocarditis; (iv) arrhythmogenic right ventricular dysplasia; (vii) hypertrophic or peripartum cardiomyopathy.

Follow-up

Patients were followed for at least 6 months after their first visit to our specialized outpatient clinic up to 12 years with a median follow-up of 45 months [interquartile range (IQR), 20–77 months]. Follow-up data on (cardiac) death, heart transplantation (HTx), life-threatening ventricular arrhythmias (LTA), and unscheduled HF hospitalization were collected using medical records, municipal population register and/or telephone contact with general practitioners. End of follow-up was defined by 1 July 2016. No patients were lost to follow-up. The primary endpoint was the combination of cardiac death, HTx, LTAs, and HF hospitalization. Life-threatening arrhythmias is defined as non-fatal ventricular fibrillation (with or without ICD-shock), haemodynamic unstable sustained ventricular tachycardia, and/or sustained ventricular tachycardia with appropriate ICD-shock.

Transthoracic echocardiography

Echocardiographic measurements were performed in the standard parasternal, apical, and subxiphoidal views at baseline and after 1 year follow-up (median 10 months; IQR 7–14 months). Follow-up echocardiography after 1 year was available in 260 (86%) patients. Left ventricular reverse remodelling (LVRR) was characterized as an absolute increase of ≥10% or LVEF ≥ 50% with at least 5% improvement. Observers of echocardiography were blinded to clinical and genetic data.

Magnetic resonance imaging

Cine and late enhancement (LE) images were acquired on a 1.5 T MRI system (Intera, Philips Medical Systems, Best, The Netherlands). Late enhancement imaging was performed 10 min after an intravenous bolus of 0.2 mmol/kg body weight gadolinium-diethylenetriaminepentaacetic acid (Gadovist, Bayer, Berlin, Germany). Two observers, blinded to genotyping data, analysed the cine and LE images by using commercially available software (CAAS MRV3.0, Pie Medical Imaging, Maastricht, The Netherlands).

Endomyocardial biopsy

At least six endomyocardial biopsies (EMB) samples were taken from the right ventricular septum via the internal jugular vein or the femoral artery using a transcatheter bioptome. Biopsies were collected as part of routine diagnostics for DCM, to identify other triggers related to DCM such as viruses, inflammation, storage diseases, and metabolic disorders. Biopsy specimens were used for the immunohistological analysis and for the detection of viral genomes (adenovirus, enterovirus, cytomegalovirus, parvovirus B19, human herpesvirus-6, and Epstein–Barr virus). Significant viral load was defined as ≥500 copies/μg DNA.[3,17] A more detailed description of the methods is in the Supplementary material online. Increased cardiac inflammation was defined as ≥14 CD45, including up to

4 CD68-infiltrating cells/mm^2 according to the current ESC guidelines.[18] Collagen fraction volume (CFV) was quantified as percentage tissue positive for Sirius red of the total myocardial area.

Genetic evaluation

All patients received genetic counselling and analysis of a 47-cardiomyopathy-associated gene-panel including *TTN* (Supplementary material online, *Table S1*). The family history of cardiac related disease and/sudden cardiac death was obtained by pedigree analysis. Familial inheritance was defined as the presence of two or more affected non-ischaemic DCM individuals in a single family.[16] The performance and interpretation of the genetic analysis is described in the Supplementary material online.

Metabolomic analysis

Metabolomic analysis of acylcarnitines and amino acids was performed in plasma samples of all DCM patients included since February 2015 using electro-spray ionization ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). Acylcarnitine profiling and amino acid quantification were performed as described previously[19].

RNA-sequencing

RNA was isolated from EMBs of selected DCM patients and checked for quality and integrity. The mRNA sequencing library was generated using TruSeq mRNA sample preparation kit (Illumina) and sequenced on the NextSeq 500 (Illumina). The bioinformatics tools Ingenuity® pathway analysis (IPA®) and GAGE [with Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment] were used for molecular pathway analysis. Metabolic pathways were used from Wikipathways and Pathvisio software to visualize the expression changes found in the data sets. The RNA sequencing steps and bioinformatical analysis are described in more detail in the Supplementary material online.

Statistical analysis

Variables are displayed as numbers (percentage), mean ± standard deviation or median (IQR) as appropriate. Categorical variables were compared using χ^2 test or Fisher's exact test. Continuous variables were compared using Student's *t*-test or Mann–Whitney *U* test. The Kaplan–Meier method was used to calculate survival curves (comparison between groups by log-rank test). Univariable Cox proportional hazards regression analysis was performed to assess clinical and demographic covariates associated with event-free survival. Statistical significance was accepted at the 95% confidence interval (95% CI) ($P < 0.05$). Statistical analysis was performed using SPSS 23.0 (IBM Corp., Armonk, NY, USA) software.

Results

Study population

Thirty-eight out of 303 patients had a TTNtv (13%) (Supplementary material online, *Figure S1* and *Table S2*). A pathogenic mutation in a different cardiomyopathy-associated gene was detected in 35 patients ($n = 12\%$; Supplementary material online, *Table S3*). None of these patients had a double pathogenic mutation. The majority of these patients were male (76%). Significantly more patients with a TTNtv had a family history of DCM as compared to the TTNtv negative patients (*Table 1*; 55% vs. 15%; $P < 0.001$).

Table 1 Baseline characteristics of dilated cardiomyopathy patients with and without a truncating titin variant

Baseline variables	TTNtv+ ($n = 38$)	TTNtv– ($n = 265$)	*P*-value
Male gender (%)	29 (76)	161 (61)	0.07
Age of diagnosis (years)	51 ± 10	52 ± 12	0.65
Family history of DCM (%)	20 (53)	40 (15)	<0.001
Hypertension (%)	13 (34)	88 (33)	0.90
Diabetes mellitus (%)	6 (16)	27 (10)	0.28
Beta blocker (%)	31 (82)	217 (82)	0.96
ACE-I/ARB (%)	35 (92)	232 (88)	0.67
MRA (%)	16 (42)	92 (35)	0.37
NYHA ≥3 (%)	10 (26)	59 (22)	0.58
Symptom duration (month)	5 (2–62)	3 (0–21)	0.09
Genetic analysis			
Pathogenic mutation (%)	38 (100)	35 (13)	<0.001
Baseline ECG/Holter			
cLBBB (%)	3 (8)	81 (31)	0.004
AV block (%)	6 (16)	19 (7)	0.16
Atrial fibrillation (%)	11 (29)	48 (18)	0.12
NSVT (%)	15 (39)	22 (8)	<0.001
Echocardiography			
LVEF (%)	32 ± 10	33 ± 11	0.55
LVEF ≤35% (%)	23 (61)	134 (51)	0.25
LVEDD (mm)	60 ± 7	60 ± 9	0.99
LVESD (mm)	50 ± 9	50 ± 11	0.91
LVEDDI (mm/m^2)	30 ± 4	30 ± 5	0.54
Cardiac MRI			
Performed (%)	31 (82)	198 (75)	0.36
LV Mi	65 ± 12	75 ± 24	0.001
LV EDVi	122 ± 32	133 ± 47	0.20
LV ESVi	85 ± 34	91 ± 49	0.58
LV SVi	39 ± 10	43 ± 11	0.06
LV EF	32 ± 10	35 ± 13	0.17
Midwall fibrosis (%)	15 (48)	85 (43)	0.62
Endomyocardial biopsy			
Performed (%)	26 (68)	215 (81)	0.09
Cardiac inflammation (%)	4 (15)	53 (25)	0.40
CD3+ (cells/mm^2)	5.99 (3.1–8.3)	5 (2.8–8.5)	0.51
CD45+ (cells/mm^2)	8 (6–10.8)	9 (5.5–13.2)	0.31
CD68+ (cells/mm^2)	2 (0.9–5.9)	2 (1.2–4.7)	0.75
Myocardial fibrosis	12.14 (7–16.5)	6.34 (3.8–9.9)	<0.001
Significant viral load			
PVB19 (%)	2 (8)	28 (13)	0.75
HHV6 (%)	0 (0)	7 (3)	0.99

Values are mean ± SD, *n*, *n* (%), or median (interquartile range).
ACE-I/ARB, angiotensin converting enzyme-inhibitor/angiotensin II receptor blocker; AV-block, atrioventricular block; cLBBB, complete left bundle branch block; DCM, dilated cardiomyopathy; HHV6, human herpesvirus 6; LVEDD, left ventricular end-diastolic diameter; LVEDDI, indexed LVEDD; LV EDVi, indexed left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; LV Mi, indexed left ventricular mass; LVESD, left ventricular end-systolic diameter; LV ESVi, indexed left ventricular end-systolic volume; LV SVi, indexed left ventricular stroke volume; MRA, mineralocorticoid receptor antagonist; NSVT, non-sustained ventricular tachycardia; PVB19, parvovirus B19.

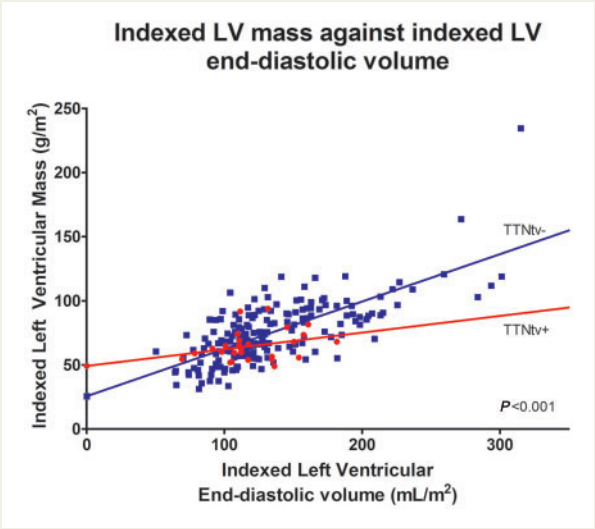


Figure 1 Indexed left ventricular mass against indexed left ventricular end-diastolic volume. Indexed left ventricular mass (LVMi) is plotted against indexed left ventricular end-diastolic volume (LVEDVi). There is a reduced increase in indexed left ventricular mass with increasing LVEDVi in truncating titin variant (TTNtv) patients (regression equations: TTNtv+: LVMi = 49.29 + 0.13 * LVEDVi vs. TTNtv-: LVMi = 26.62 + 0.37 * LVEDVi; $P < 0.001$).

Lower left ventricular mass in truncating titin variant patients

Left ventricular ejection fraction and/or left ventricular end-diastolic/systolic diameter did not differ between the two groups at baseline (*Table 1*). However, indexed left ventricular mass (LVMi) was significantly reduced in TTNtv patients despite the absence of differences in left ventricular end diastolic volume (LVEDVi) (*Table 1*). As LVMi normally increases with increasing LVEDVi, the increase of LVMi per 1 mL/m^2 LVEDVi is significantly reduced in TTNtv patients [0.13 g/m^2 in TTNtv vs. 0.37 g/m^2; $P < 0.001$ (*Figure 1*)].

Enhanced interstitial myocardial fibrosis and arrhythmias at baseline in truncating titin variant patients

Interstitial myocardial fibrosis at EMB was significant higher in patients with a TTNtv (*Figure 2*; 12% vs. 6.3%; $P < 0.001$), whereas mid-wall replacement fibrosis at CMR did not differ among the groups (*Table 1*). The interstitial fibrosis at EMBs had a homogeneous intramyocardial pattern, suggestive of reactive fibrosis (*Figure 2*; Supplementary material online, *Figure S2*).

Increased interstitial myocardial fibrosis went along with a slightly higher incidence of non-sustained ventricular tachycardias (NSVT) at baseline (Supplementary material online, *Figure S3*; $P = 0.04$). Notably, TTNtv patients had more NSVT at early presentation (*Table 1*; $P < 0.001$). The latter was also reflected by a higher incidence of (pre)syncope and/or palpitations in TTNtv patients. This interstitial myocardial fibrosis at EMB did not correlate with the focal mid-wall fibrosis measured by CMR LE imaging ($P = 0.11$). In conclusion, these data revealed enhanced interstitial myocardial fibrosis at EMBs but decreased mass of TTNtv hearts, along with increased NSVTs and related symptoms at baseline.

More life threatening arrhythmias but similar survival at long-term follow-up

The rate of LVRR did not significantly differ between the groups at 1-year echocardiographic follow-up (*Figure 3*).

A total of 51 patients (17%) reached the primary endpoint during a median follow-up of 45 months (IQR 20–77 months): cardiac death ($n = 12$), Htx ($n = 2$), LTA ($n = 28$), or HF hospitalization ($n = 18$). Short- and long-term outcome, survival, and the combined primary endpoints did not significantly differ between the groups (*Table 2*; *Figure 4A*). However, TTNtv patients had a significantly higher risk of a LTA at long-term follow-up, mainly sustained VT [9 (24%) vs. 19 (7%); HR 2.78; 95% CI 1.23–6.29; $P = 0.014$; *Table 2*; *Figure 4B*]. The occurrence of a LTA significantly correlated with the presence of NSVT at baseline ($P < 0.001$; HR 5.27; 95% CI 2.46–11.29).

Gene–environmental interactions in truncating titin variant patients

Patients were classified according to their possible contributing environmental factors to score the gene–environmental interactions in TTNtv patients as described before (Supplementary material online, *Table S4*).[3] Seventeen TTNtv patients (45%) had an environmental factor possible contributing to their phenotype (i.e. viral, immune-mediated, toxic). Truncating titin variant patients with an additional environmental factor have a significantly higher risk of developing LTA at long-term (log-rank = 0.047; *Figure 5*). However, due to the low number of TTNtv patients, these results should be considered as hypothesis-generating.

Alteration of metabolic and structural pathways in the heart of truncating titin variant patients

To gain better insight into the cardiac pathophysiological changes in response to TTNtv, genome-wide transcriptome analysis (RNA-sequencing of EMB) was performed in a representative group of patients with similar HF severity, as defined by LVEF and NYHA class ($n = 21$, 13 TTNtv+, 8 TTNtv- DCM; Supplementary material online, *Table S5* and *Figure S4*). In general, RNA sequencing showed comparable *TTN* transcript levels in patients with or without TTNtv (Supplementary material online, *Table S6*). Subsequent gene set enrichment analysis with KEGG terms using the GAGE bioinformatics tool, revealed significant alterations of different molecular pathways in TTNtv patients compared to the other HF group (*Figure 6A*). Many of the significantly enriched pathways were related to cardiac energy metabolism. Differential expressed genes were analysed with the IPA® software to further validate these findings. Here, mitochondrial dysfunction was most affected (*Table 3*). Notably, closer visualization on the mitochondrial oxidative phosphorylation pathway by means of Pathvisio and Wikipathways revealed increased expression of genes across all the electron transport chain complexes (ETC), involving complex I to IV as well as the adenosine triphosphate (ATP) synthase (complex V) (*Figure 6B*). Remarkably, the marked changes in ETC were not associated with concurrent changes in the expression of enzymes involved in the citric acid cycle,

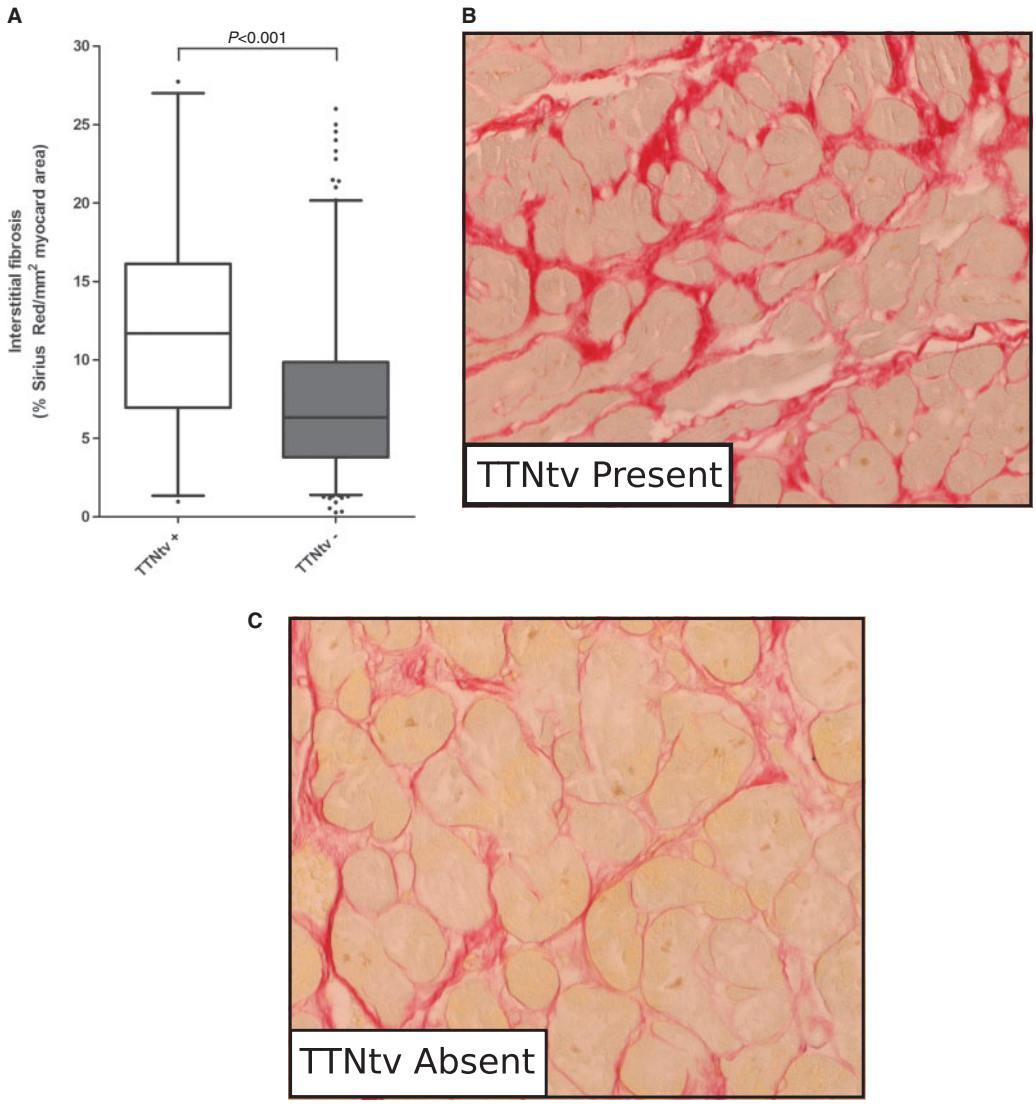


Figure 2 Interstitial myocardial fibrosis in dilated cardiomyopathy. Interstitial fibrosis quantified in the endomyocardial biopsies of patients with and without a truncating titin variant (*A*). Boxes represent the median and interquartile range, whiskers are 5–95% range, dots represent the outliers. Representative pictures of Sirius red stainings on endomyocardial biopsies of a truncating titin variant (*B*) and a patient without truncating titin variant (*C*). Magnification 200×.

fatty acid oxidation, or glycolysis (Supplementary material online, *Figures S5–S7*). Next to alterations in the ETC, perturbations in cardiac hypertrophy, oxidative stress and fibrosis in the TTNtv heart are suggested by gene set enrichment analysis showing overlapping KEGG and IPA® terms (*Figure 6A*; *Table 3*).

To further investigate the metabolic consequence of altered gene expression in TTNtv patients; amino acids, acylcarnitines, and very-long-chain fatty acids were measured in plasma (Supplementary material online, *Table S7*). Relative to the other group, short- and medium-chain acyl-carnitines, and very-long-chain fatty acids were elevated in the plasma of TTNtv patients (Supplementary material online, *Figure S8* and *Table S8*). The principal long-chain acylcarnitine (C16: 0, C18: 0, C18: 1 and C18: 2) did not differ. Also, plasma branched-chain amino acids (BCAA) were not elevated in TTNtv patients (Supplementary material online, *Figure S9*). All metabolic analyses were corrected for gender, as all TTNtv patients were male (Supplementary material online, *Table S7*).

Discussion

This study examined the phenotypic characteristics and clinical outcomes at baseline and after long-term follow-up of TTNtv DCM patients and unveiled the underlying cardiac molecular changes. In TTNtv DCM hearts, pronounced cardiac alterations in mitochondrial function, increased interstitial fibrosis and reduced ventricular mass was found. Those structural and metabolic alterations related to

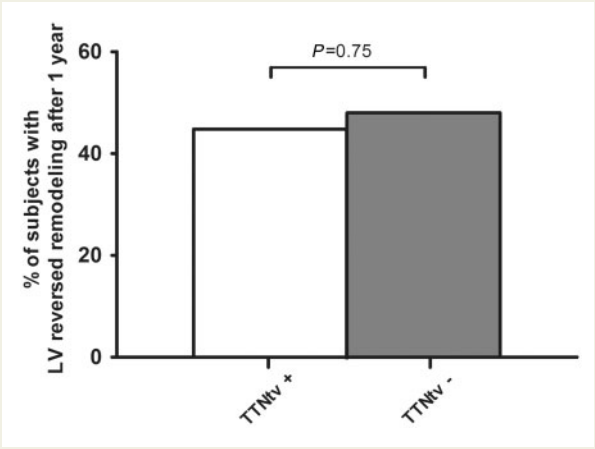


Figure 3 Left ventricular reverse remodelling in dilated cardiomyopathy. Percentage of patients who improved or/and recovered after 1 year measured using echocardiographic follow-up data. Improvement is defined as an absolute increase of ≥10% or a left ventricular ejection fraction ≥ 50% with at least 5% improvement. LV, left ventricular.

Table 2 Primary endpoint and hazard ratios

	TTNtv+ ($n = 38$)	TTNtv− ($n = 265$)	*P*-value	Gender-adjusted hazard ratio (95% CI)
Follow-up duration (months)	39 [16–87]	46 [22–76]	0.84	—
Death	0 (0)	12 (5)	0.37	—
Heart transplantation	0 (0)	2 (1)	0.99	—
HF hospitalization	3 (8)	15 (6)	0.48	1.20 (0.34–4.25)
Life-threatening arrhythmia	9 (24)	19 (7)	0.004	2.78 (1.23–6.29)
Combined endpoint	10 (26)	41 (15)	0.10	1.52 (0.75–3.08)

Values are *n*, *n* (%), or median (interquartile range).
CI, confidence interval; HF, heart failure.

TTNtv went along with an increased propensity to (life-threatening) ventricular arrhythmias at long-term follow-up (*Take home figure*).

In our cohort, the prevalence of TTNtv mutations was 13% (33% in familial DCM and 7% in sporadic cases) in line with previous studies.[4,20,21] Structural analysis of EMB from living TTNtv patients revealed enhanced myocardial interstitial fibrosis as compared to TTNtv negative DCM patients. Fibrosis is known to be a substrate for arrhythmogenesis in DCM patients,[22] and may therefore in part explain the ventricular arrhythmogenic burden in TTNtv patients at long-term follow-up found here, and by others at baseline.[5,9,10] The degree of late enhancement at CMR did not differ in TTNtv compared to TTNtv negative DCM patients, as observed previously.[5] This paradox between histological fibrosis and CMR late enhancement is in line with the rather reactive diffuse interstitial fibrosis at EMB upon pathogenic *TTN* gene mutation, which cannot be detected

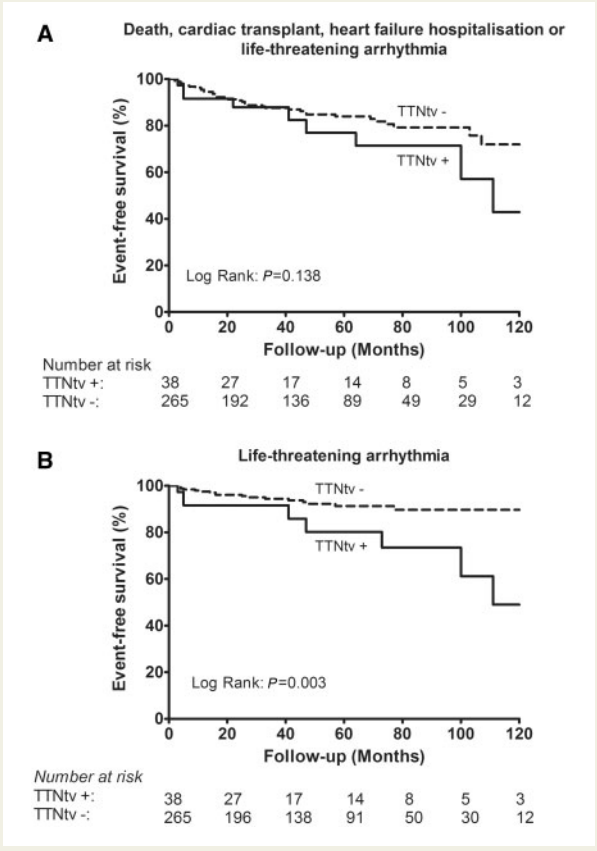


Figure 4 Truncating titin variant and long-term outcome in dilated cardiomyopathy. Primary outcomes in TTNtv+ and TTNtv- dilated cardiomyopathy patients (*A*). Primary endpoint after enrolment (cardiac death, Htx, heart failure hospitalization or life-threatening arrhythmia) (*B*). Occurrence of a life-threatening arrhythmia after enrolment. Event-free survival regarding life-threatening arrhythmia is reduced in truncating titin variant patients.

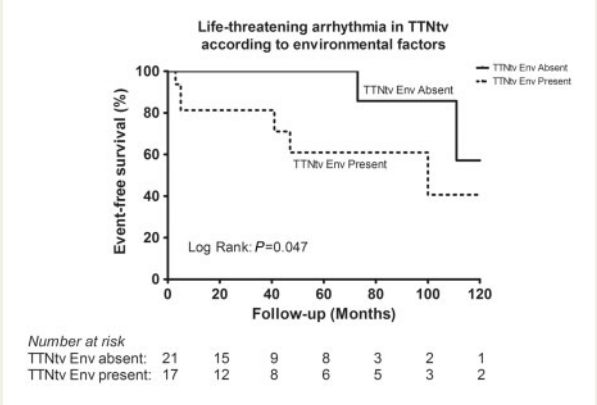


Figure 5 Life-threatening arrhythmia in truncating titin variant according to environmental factors. The occurrence of a life-threatening arrhythmia after enrolment. There is significant reduction in event-free survival regarding life-threatening arrhythmia in truncating titin variant patients ($P = 0.047$). Truncating titin variant. Env absent, absence of an environmental factor; Env Present, presence of an environmental factor; TTNtv, truncating titin variant.

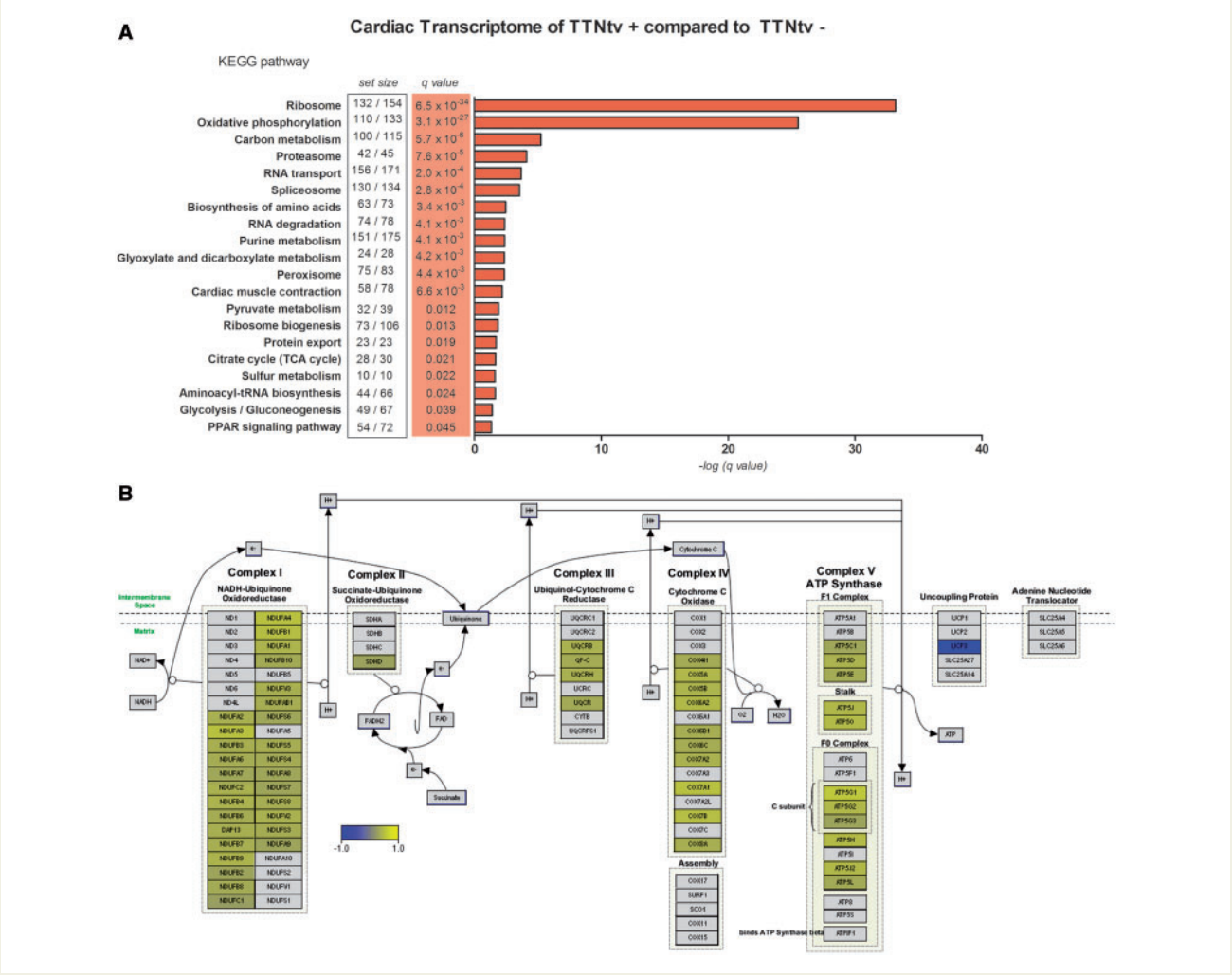


Figure 6 Molecular pathway analysis in endomyocardial biopsies of truncating titin variant dilated cardiomyopathy patients. Pathway analysis based on RNA-seq data of endomyocardial biopsies from dilated cardiomyopathy patients show altered metabolism and structural integrity in truncating titin variant patients (*A*). Significantly enriched pathways are highlighted in red (*q*-value <0.05). Set size is the number of found genes in the RNA-seq data in relation to the total genes in a KEGG pathway. The top enriched oxidative phosphorylation pathway is graphically depicted using Wikipathways and Pathvisio (*B*). Yellow shows significant up-regulation of a gene. ATP, adenosine triphosphate; KEGG, Kyoto Encyclopaedia of Genes and Genomes; PPAR, peroxisome proliferator-activated receptor; TCA, tricarboxylic acid.

Table 3 Top five differentially expressed biological pathways using Ingenuity pathway analysis (IPA) between TTNtv+ and TTNtv− patients

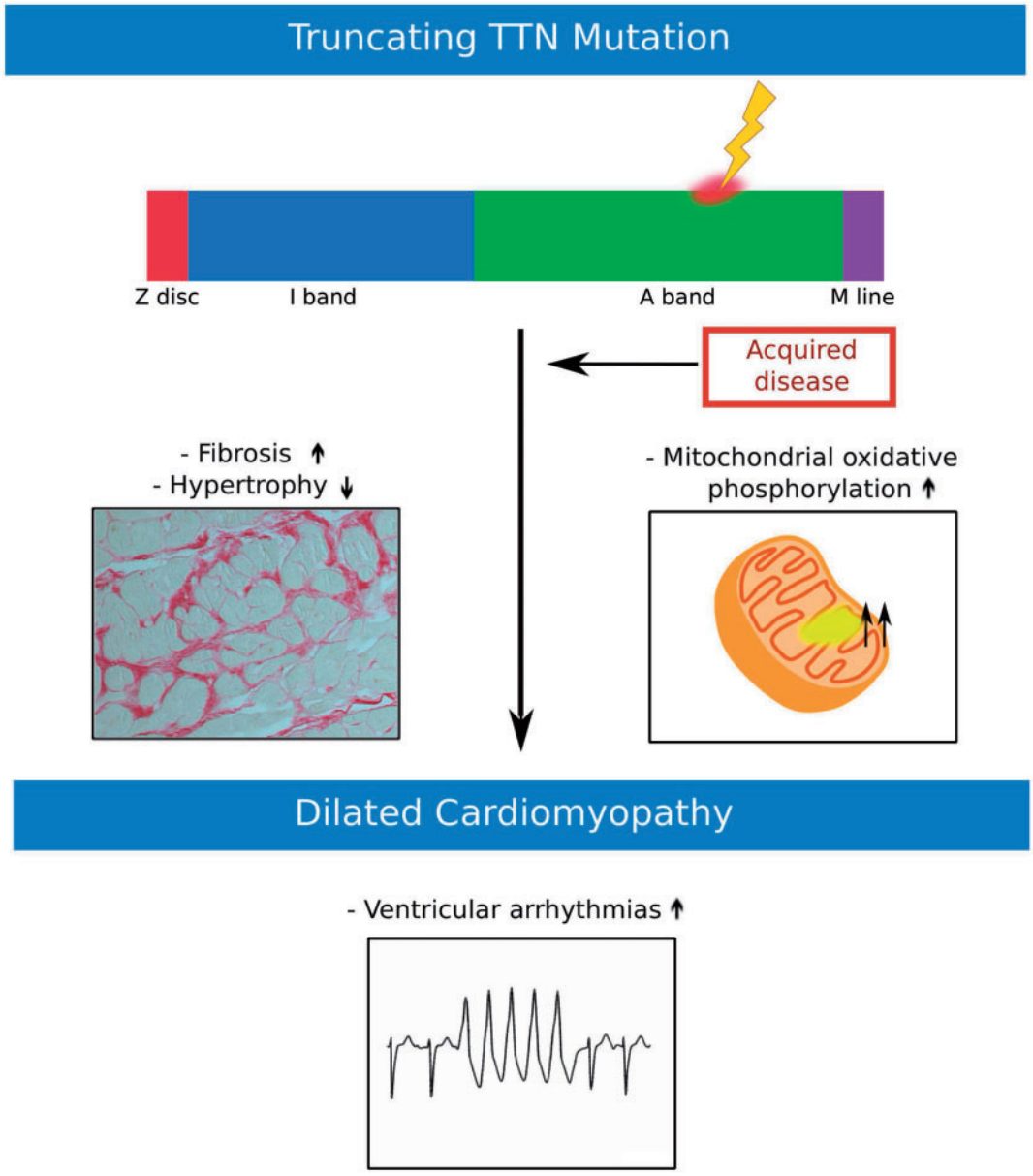
Ingenuity toxicity list[a]	*P*-value	% genes[b]
TTNtv+ vs. TTNtv-		
Mitochondrial dysfunction	1.58E-21	35% (62/176)
Cardiac hypertrophy	4.57E-03	13% (44/342)
Increases renal nephritis	0.02	19% (11/58)
NRF2-mediated oxidative stress response	0.02	13% (27/216)
Cardiac fibrosis	0.03	13% (27/207)

[a]1.796 significantly differentially expressed transcripts were considered for pathway analysis.
[b]Percentage of pathway-related genes that are differentially expressed between the data sets followed by the absolute number.

with late enhancement CMR, the latter only sensitive for extensive replacement fibrosis.[23] Despite the increased interstitial fibrosis, TTNtv patients seem to respond well to therapy, possibly due to the reactive character of the fibrosis in the absence of cardiomyocyte death. Whether T1 mapping—not routinely performed in our centre before 2016—may be helpful to quantify this diffuse interstitial fibrosis requires further investigation.

More life-threatening arrhythmias but a similar prognosis in truncating titin variant dilated cardiomyopathy patients

Our patients with a TTNtv had more ventricular arrhythmias at baseline and LTAs at long-term, mainly in those with additional environmental factors, suggesting a more severe clinical course when a second (environmental) trigger is present on top of the TTNtv. This



Take home figure Truncating *TTN* mutations and Dilated Cardiomyopathy. Truncating mutations in the *TTN* gene can lead to DCM associated with ventricular arrhythmias, increased interstitial fibrosis, lower ventricular mass and alterations of the mitochondrial energy pathways.

first insight into the importance of gene–environmental interaction in the LTA of our well-phenotyped TTNtv patients, is in line with similar observations with the phenotypic and prognostic impact of gene–environmental interaction in other DCM cohorts.[3,24]

Despite the arrhythmogenic burden, we did not observe a significant impact on prognosis (i.e. death, heart transplantation), potentially due to effective device therapy (ICD). Also, almost 50% of our TTNtv patients have significant LVRR after 1-year HF medication. TTNtv DCM thus seems to respond well to HF therapy in line with previous observations,[9] but still are more prone to ventricular arrhythmias at long-term. In the latest ESC guidelines, *LMNA* mutations are the only mutations for which preventive ICD therapy should be considered in DCM.[25] However, the current and other studies indicate that TTNtv should also be considered as an additional genetic risk for ventricular arrhythmias.[5,10]

Pronounced mitochondrial remodelling in truncating titin variant dilated cardiomyopathy patients

The consistently higher transcript levels of all mitochondrial electron transport chain (ETC) complexes in TTNtv hearts is remarkable. Especially while other building blocks of cardiac energy metabolism (citric acid cycle, fatty acid oxidation, glycolysis) are relatively unaffected. These findings suggest an increased efficiency of mitochondrial

ATP production trying to counterbalance a TTNtv-related sarcomeric defect. It may thus represent an attempt to compensate for the limited contractile and reserve capacity in TTNtv mutations previously observed *in vitro*.[26] This finding supports the suggestion that TTNtv hearts are in a compensated state inflexible to further stress as suggested by others.[6]

Our cardiac metabolic data are in line with the metabolic remodelling found in TTNtv mutated rat hearts compared to control hearts recently reported.[6] Similar to our patients, TTNtv mutated rat hearts had an enrichment of mitochondrial pathways and increased transcript levels of respiratory chain constituents (*Atp1a3, Atp6ap1*). Importantly, these TTNtv rats were compared to healthy wild type hearts. The latter suggests that the human cardiac changes we observe in TTNtv patients reflect true up-regulation of mitochondrial energetics and not less down-regulation compared to TTNtv negative DCM patients. The direct causal links by which these *TTN* gene mutations lead to mitochondrial alterations require further investigation e.g. *in vitro*, animal or humanized cell models, to understand the significance from a clinical and therapeutic perspective.

In HF, it is generally acknowledged that mitochondrial oxidative energy metabolism becomes impaired and is compensated by an increased anaerobic glycolysis.[11] This translates in elevated long-chain acylcarnitines levels in the circulation of these patients. Also, BCAA are often elevated in HF patients, although reasons for this increase are unknown. However, plasma levels of small- and medium chain acylcarnitines were increased in our TTNtv patients, but circulating levels of long-chain acylcarnitines were unaltered. Elevated blood long-chain acylcarnitines have been independently associated with arrhythmias and adverse clinical outcomes.[12] The unaltered level of long-chain acylcarnitines does not support the idea that cardiac mitochondrial function is severely compromised in TTNtv patients.[11] Instead, the massive up-regulation of respiratory chain transcripts may indeed point to a compensatory increase in mitochondrial function. Therefore, the increased propensity for LTAs in TTNtv patients is not driven by an accumulation of noxious lipid intermediates in blood.

Increased cardiac fibrosis, pronounced energetic alterations and increased ventricular arrhythmias at long-term were associated with a decreased LV cardiac mass in TTNtv patients, whereas cardiac systolic function or dimensions did not differ. Although this decreased cardiac mass is not directly clinically relevant, it offers insight in the pathomechanism of TTNtv DCM. Whether this reduction in cardiac mass relates to a lower ability for hypertrophic compensation to (mechanical) stress (as titin is important for mechanotransduction at the sarcomere), or whether less cardiomyocyte hypertrophy is needed to compensate for this gene deficit compared to others, requires further investigations.

Limitations

The current study population was enrolled in a single tertiary referral centre, thus imposing a possible selection bias. Because of the relative low number of TTNtv patients in our cohort, the current study had insufficient power to perform multivariable modelling. Although the results are clear and unidirectional, sub-analyses (such as the gene-environmental analysis) lack adequate power and should be considered as hypothesis-generating. Therefore, these results should be interpreted with caution and need confirmation in larger cohorts.

Conclusions

In summary, DCM patients with a TTNtv are prone to ventricular arrhythmias at long term, which is aggravated in combination with an additional environmental factor. These arrhythmias go hand in hand with increased cardiac interstitial fibrosis and pronounced changes in mitochondrial function.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

Acknowledgements

We acknowledge the support from the Netherlands Cardiovascular Research Initiative: an initiative with support of the Dutch Heart Foundation, CVON2011-ARENA, CVON2016-Early HFPEF and CVON 2017-ShePREDICTS. This research is co-financed as a PPP-allowance Research and Innovation by the Ministry of Economic Affairs within Top Sector Life sciences & Health.

Funding

European Union Commission's Seventh Framework programme under [Grant Agreement No. 305507] (HOMAGE); and FP7-Health-2013-Innovations-1 [No. 602156] (HECATOS); Kootstra Talent Fellowship of the Maastricht UMC+ to J.V.

Conflict of interest: none declared.

References

1. Hershberger RE, Hedges DJ, Morales A. Dilated cardiomyopathy: the complexity of a diverse genetic architecture. *Nat Rev Cardiol* 2013;**10**:531–547.
2. Merlo M, Pivetta A, Pinamonti B, Stolfo D, Zecchin M, Barbati G, Di Lenarda A, Sinagra G. Long-term prognostic impact of therapeutic strategies in patients with idiopathic dilated cardiomyopathy: changing mortality over the last 30 years. *Eur J Heart Fail* 2014;**16**:317–324.
3. Hazebroek MR, Moors S, Dennert R, van den Wijngaard A, Krapels I, Hoos M, Verdonschot J, Merken JJ, de Vries B, Wolffs PF, Crijns HJ, Brunner-La Rocca HP, Heymans S. Prognostic relevance of gene-environment interactions in patients with dilated cardiomyopathy: applying the MOGE(S) classification. *J Am Coll Cardiol* 2015;**66**:1313–1323.
4. Haas J, Frese KS, Peil B, Kloos W, Keller A, Nietsch R, Feng Z, Muller S, Kayvanpour E, Vogel B, Sedaghat-Hamedani F, Lim WK, Zhao X, Fradkin D, Kohler D, Fischer S, Franke J, Marquart S, Barb I, Li DT, Amr A, Ehlermann P, Mereles D, Weis T, Hassel S, Kremer A, King V, Wirsz E, Isnard R, Komajda M, Serio A, Grasso M, Syrris P, Wicks E, Plagnol V, Lopes L, Gadgaard T, Eiskjaer H, Jorgensen M, Garcia-Giustiniani D, Ortiz-Genga M, Crespo-Leiro MG, Deprez RH, Christiaans I, van Rijsingen IA, Wilde AA, Waldenstrom A, Bolognesi M, Bellazzi R, Morner S, Bermejo JL, Monserrat L, Villard E, Mogensen J, Pinto YM, Charron P, Elliott P, Arbustini E, Katus HA, Meder B. Atlas of the clinical genetics of human dilated cardiomyopathy. *Eur Heart J* 2015;**36**:1123–135a.
5. Roberts AM, Ware JS, Herman DS, Schafer S, Baksi J, Bick AG, Buchan RJ, Walsh R, John S, Wilkinson S, Mazzarotto F, Felkin LE, Gong S, MacArthur JA, Cunningham F, Flannick J, Gabriel SB, Altshuler DM, Macdonald PS, Heinig M, Keogh AM, Hayward CS, Banner NR, Pennell DJ, O'regan DP, San TR, de Marvao A, Dawes TJ, Gulati A, Birks EJ, Yacoub MH, Radke M, Gotthardt M, Wilson JG, O'donnell CJ, Prasad SK, Barton PJ, Fatkin D, Hubner N, Seidman JG, Seidman CE, Cook SA. Integrated allelic, transcriptional, and phenomic dissection of the cardiac effects of titin truncations in health and disease. *Sci Transl Med* 2015;**7**:270ra6.
6. Schafer S, de Marvao A, Adami E, Fiedler LR, Ng B, Khin E, Rackham OJ, van Heesch S, Pua CJ, Kui M, Walsh R, Tayal U, Prasad SK, Dawes TJ, Ko NS, Sim D, Chan LL, Chin CW, Mazzarotto F, Barton PJ, Kreuchwig F, de Kleijn DP, Totman T, Biffi C, Tee N, Rueckert D, Schneider V, Faber A, Regitz-Zagrosek V, Seidman JG, Seidman CE, Linke WA, Kovalik JP, O'regan D, Ware JS, Hubner N, Cook SA. Titin-truncating variants affect heart function in disease cohorts and the general population. *Nat Genet* 2017;**49**:46–53.
7. Linschoten M, Teske AJ, Baas AF, Vink A, Dooijes D, Baars HF, Asselbergs FW. Truncating titin (TTN) variants in chemotherapy-induced cardiomyopathy. *J Card Fail* 2017;**23**:476–479.

8. van Spaendonck-Zwarts KY, Posafalvi A, van den Berg MP, Hilfiker-Kleiner D, Bollen IA, Sliwa K, Alders M, Almomani R, van Langen IM, van der Meer P, Sinke RJ, van der Velden J, Van Veldhuisen DJ, van Tintelen JP, Jongbloed JD. Titin gene mutations are common in families with both peripartum cardiomyopathy and dilated cardiomyopathy. *Eur Heart J* 2014;**35**:2165–2173.
9. Jansweijer JA, Nieuwhof K, Russo F, Hoorntje ET, Jongbloed JD, Lekanne Deprez RH, Postma AV, Bronk M, van Rijsingen IA, de Haij S, Biagini E, van Haelst PL, van Wijngaarden J, van den Berg MP, Wilde AA, Mannens MM, de Boer RA, van Spaendonck-Zwarts KY, van Tintelen JP, Pinto YM. Truncating titin mutations are associated with a mild and treatable form of dilated cardiomyopathy. *Eur J Heart Fail* 2017;**19**:512–521.
10. Tayal U, Newsome S, Buchan R, Whiffin N, Walsh R, Barton PJ, Ware JS, Cook SA, Prasad SK. Truncating variants in titin independently predict early arrhythmias in patients with dilated cardiomyopathy. *J Am Coll Cardiol* 2017;**69**:2466–2468.
11. Ussher JR, Elmariah S, Gerszten RE, Dyck JR. The emerging role of metabolomics in the diagnosis and prognosis of cardiovascular disease. *J Am Coll Cardiol* 2016;**68**:2850–2870.
12. Ahmad T, Kelly JP, McGarrah RW, Hellkamp AS, Fiuzat M, Testani JM, Wang TS, Verma A, Samsky MD, Donahue MP, Ilkayeva OR, Bowles DE, Patel CB, Milano CA, Rogers JG, Felker GM, O'connor CM, Shah SH, Kraus WE. Prognostic implications of long-chain acylcarnitines in heart failure and reversibility with mechanical circulatory support. *J Am Coll Cardiol* 2016;**67**:291–299.
13. Alexander D, Lombardi R, Rodriguez G, Mitchell MM, Marian AJ. Metabolomic distinction and insights into the pathogenesis of human primary dilated cardiomyopathy. *Eur J Clin Invest* 2011;**41**:527–538.
14. Ponikowski P, Voors AA, Anker SD, Bueno H, Cleland JG, Coats AJ, Falk V, Gonzalez-Juanatey JR, Harjola VP, Jankowska EA, Jessup M, Linde C, Nihoyannopoulos P, Parissis JT, Pieske B, Riley JP, Rosano GM, Ruilope LM, Ruschitzka F, Rutten FH, van der Meer P. Authors/Task Force M. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC) Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. *Eur Heart J* 2016;**37**:2129–2200.
15. Merlo M, Pyxaras SA, Pinamonti B, Barbati G, Di Lenarda A, Sinagra G. Prevalence and prognostic significance of left ventricular reverse remodeling in dilated cardiomyopathy receiving tailored medical treatment. *J Am Coll Cardiol* 2011;**57**:1468–1476.
16. Pinto YM, Elliott PM, Arbustini E, Adler Y, Anastasakis A, Bohm M, Duboc D, Gimeno J, de Groote P, Imazio M, Heymans S, Klingel K, Komajda M, Limongelli G, Linhart A, Mogensen J, Moon J, Pieper PG, Seferovic PM, Schueler S, Zamorano JL, Caforio AL, Charron P. Proposal for a revised definition of dilated cardiomyopathy, hypokinetic non-dilated cardiomyopathy, and its implications for clinical practice: a position statement of the ESC working group on myocardial and pericardial diseases. *Eur Heart J* 2016;**37**:1850–1858.
17. Verdonschot J, Hazebroek M, Merken J, Debing Y, Dennert R, Brunner-La Rocca HP, Heymans S. Relevance of cardiac parvovirus B19 in myocarditis and dilated cardiomyopathy: review of the literature. *Eur J Heart Fail* 2016;**18**:1430–1441.
18. Caforio AL, Pankuweit S, Arbustini E, Basso C, Gimeno-Blanes J, Felix SB, Fu M, Helio T, Heymans S, Jahns R, Klingel K, Linhart A, Maisch B, McKenna W, Mogensen J, Pinto YM, Ristic A, Schultheiss HP, Seggewiss H, Tavazzi L, Thiene G, Yilmaz A, Charron P, Elliott PM; European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: a position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases. *Eur Heart J* 2013;**34**:2636–2648. 2648a–2648d.
19. Waterval WA, Scheijen JL, Ortmans-Ploemen MM, Habets-van der Poel CD, Bierau J. Quantitative UPLC-MS/MS analysis of underivatised amino acids in body fluids is a reliable tool for the diagnosis and follow-up of patients with inborn errors of metabolism. *Clin Chim Acta* 2009;**407**:36–42.
20. Akinrinade O, Ollila L, Vattulainen S, Tallila J, Gentile M, Salmenpera P, Koillinen H, Kaartinen M, Nieminen MS, Myllykangas S, Alastalo TP, Koskenvuo JW, Helio T. Genetics and genotype-phenotype correlations in Finnish patients with dilated cardiomyopathy. *Eur Heart J* 2015;**36**:2327–2337.
21. Herman DS, Lam L, Taylor MR, Wang L, Teekakirikul P, Christodoulou D, Conner L, DePalma SR, McDonough B, Sparks E, Teodorescu DL, Cirino AL, Banner NR, Pennell DJ, Graw S, Merlo M, Di Lenarda A, Sinagra G, Bos JM, Ackerman MJ, Mitchell RN, Murry CE, Lakdawala NK, Ho CY, Barton PJ, Cook SA, Mestroni L, Seidman JG, Seidman CE. Truncations of titin causing dilated cardiomyopathy. *N Engl J Med* 2012;**366**:619–628.
22. Gulati A, Jabbour A, Ismail TF, Guha K, Khwaja J, Raza S, Morarji K, Brown TD, Ismail NA, Dweck MR, Di Pietro E, Roughton M, Wage R, Daryani Y, O'hanlon R, Sheppard MN, Alpendurada F, Lyon AR, Cook SA, Cowie MR, Assomull RG, Pennell DJ, Prasad SK. Association of fibrosis with mortality and sudden cardiac death in patients with nonischemic dilated cardiomyopathy. *JAMA* 2013;**309**:896–908.
23. Mewton N, Liu CY, Croisille P, Bluemke D, Lima JA. Assessment of myocardial fibrosis with cardiovascular magnetic resonance. *J Am Coll Cardiol* 2011;**57**:891–903.
24. Ware JS, Li J, Mazaika E, Yasso CM, DeSouza T, Cappola TP, Tsai EJ, Hilfiker-Kleiner D, Kamiya CA, Mazzarotto F, Cook SA, Halder I, Prasad SK, Pisarcik J, Hanley-Yanez K, Alharethi R, Damp J, Hsich E, Elkayam U, Sheppard R, Kealey A, Alexis J, Ramani G, Safirstein J, Boehmer J, Pauly DF, Wittstein IS, Thohan V, Zucker MJ, Liu P, Gorcsan J 3rd, McNamara DM, Seidman CE, Seidman JG, Arany Z; IMAC-2 and IPAC Investigators. Shared genetic predisposition in peripartum and dilated cardiomyopathies. *N Engl J Med* 2016;**374**:233–241.
25. Priori SG, Blomström-Lundqvist C, Mazzanti A, Blom N, Borggrefe M, Camm J, Elliott PM, Fitzsimons D, Hatala R, Hindricks G, Kirchhof P, Kjeldsen K, Kuck K-H, Hernandez-Madrid A, Nikolaou N, Norekvål TM, Spaulding C, Van Veldhuisen DJ. 2015 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: The Task Force for the Management of Patients with Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death of the European Society of Cardiology (ESC). Endorsed by: Association for European Paediatric and Congenital Cardiology (AEPC). *Eur Heart J* 2015;**36**:2793–2867.
26. Hinson JT, Chopra A, Nafissi N, Polacheck WJ, Benson CC, Swist S, Gorham J, Yang L, Schafer S, Sheng CC, Haghighi A, Homsy J, Hubner N, Church G, Cook SA, Linke WA, Chen CS, Seidman JG, Seidman CE. HEART DISEASE. Titin mutations in iPS cells define sarcomere insufficiency as a cause of dilated cardiomyopathy. *Science* 2015;**349**:982–986.