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CLINICAL RESEARCH
Genetics

Titin-related familial dilated cardiomyopathy: factors associated with disease onset

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Abstract

Background and Aims Truncating variants in the *TTN* gene (*TTN*tv) are the most common genetic cause of dilated cardiomyopathy (DCM) but also occur as incidental findings in the general population. This study investigated factors associated with the clinical manifestation of *TTN*tv.

Methods An international multicentre retrospective observational study was performed in families with *TTN*tv-related DCM. Shared frailty models were used to estimate associations of variant characteristics with lifetime risk of DCM, and logistic regression to estimate odds ratios (ORs) for individual-level clinical risk factor profiles (cardiac conditions, cardiovascular comorbidities, lifestyle) and DCM.

Results A total of 3158 subjects in 1043 families with *TTN*tv-related DCM were studied. *TTN*tv-positive subjects were 21-fold more likely to develop DCM [OR, 21.21; 95% confidence interval (CI), 14.80–30.39]. Disease onset was earlier in males, but was similar for *TTN*tv of different types and locations. The presence of clinical risk factors was associated with earlier DCM onset (OR, 3.41; 95% CI, 2.06–5.64), with a prior history of atrial fibrillation having a two-fold increased odds of DCM (OR, 2.05; 95% CI, 1.27–3.32). The prevalence of clinical risk factors increased with age; however, the strength of the DCM association was greatest for young-onset (<30 years) disease (OR, 4.75; 95% CI, 2.35–9.60). Administration of beta-adrenergic receptor or renin-angiotensin system-blocking drugs prior to overt DCM was associated with 87% reduced odds of DCM (OR, .13; 95% CI, .08–.23).

Conclusions Disease onset in *TTN*tv-associated familial DCM is dependent on individual patient context and is potentially modifiable by risk factor management and prophylactic therapeutic intervention.

Structured Graphical Abstract

Key Question

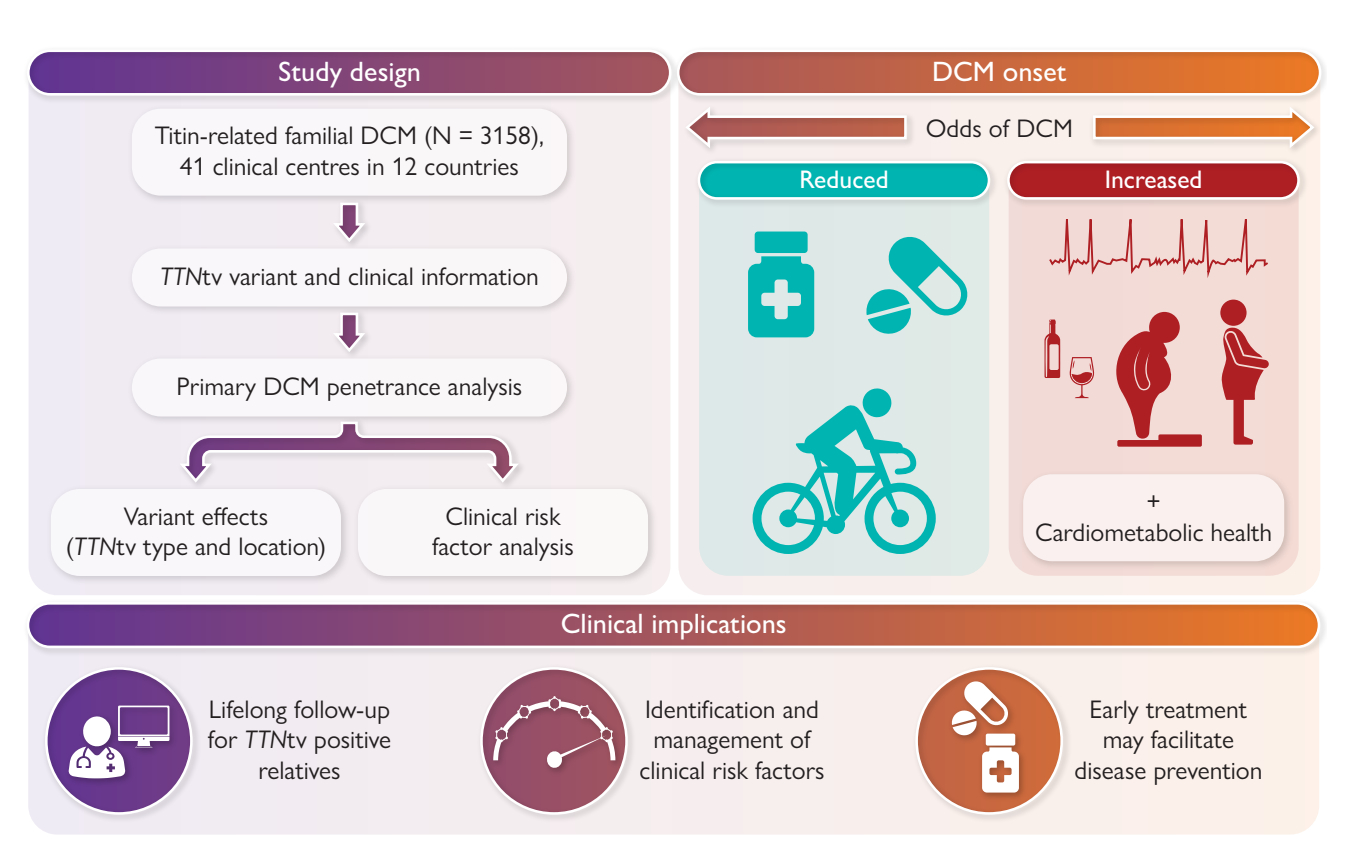
Truncating variants in the *TTN* gene (*TTN*tv) are the most common genetic cause of familial dilated cardiomyopathy (DCM) but the age at disease onset is highly variable. Is this explained by distinctive *TTN*tv characteristics or factors related to the individual patient environment?

Key Finding

In families with *TTN*tv, the lifetime risk of DCM was high, irrespective of variant type or location. DCM was diagnosed at an earlier age in *TTN*tv-positive subjects with clinical risk factors and manifested later in those receiving pharmacological therapy pre-DCM.

Take Home Message

Clinical manifestations of *TTN*tv is not solely genetically determined and is influenced by risk factors. *TTN*tv-positive members of families with DCM need ongoing cardiac screening and aggressive risk factor management. Prophylactic therapeutic intervention may be beneficial but warrants formal evaluation.



Factors associated with disease onset in titin-related familial dilated cardiomyopathy and implications for management.

Keywords Dilated cardiomyopathy • Titin • Genetics • Risk factors • Prevention

Introduction

Dilated cardiomyopathy (DCM) is a highly prevalent myocardial disorder that is associated with substantial morbidity and mortality. Understanding the aetiology of DCM in individual patients should facilitate effective disease treatment and prevention. A person's genetic makeup is a key determinant of susceptibility to DCM but to date, genotype-based approaches to clinical management have been limited.[1]

Truncating variants in the *TTN* gene (*TTN*tv) that encodes the giant sarcomere protein titin are the most common genetic cause of DCM, being present in 10%–20% of sporadic cases and up to 25% of families.[2,3] Potentially deleterious *TTN*tv in constitutively expressed exons occur in up to 1% of the general population and are often detected as incidental findings during genetic testing.[2,3] A number of criteria have been proposed to select the subset of *TTN*tv that are most likely to have clinical significance, with key features being location in the titin A-band or in exons that are constitutively

expressed across the range of titin isoforms.[2–4] However even for subjects with the same *TTN*tv, the age at DCM diagnosis can be highly variable.[5,6] Elucidating factors that accelerate or delay disease manifestation holds promise for shifting DCM onset, potentially by decades.

Uncertainty about the prognostic implications of *TTN*tv has been a major roadblock for informed genetic counselling and family management. Given the rapidly rising number of *TTN*tv-positive individuals identified by genetic testing, resolving this question has become a clinical imperative. The impact of *TTN*tv on cardiac function is likely influenced by each patient's context of background genetic factors, comorbidities, and lifestyle,[7–9] but definitive evidence for this has been lacking. To start to address these issues, we assembled a unique international multicentre cohort to investigate factors that influence DCM onset in families with *TTN*tv-related DCM.

Methods

Study cohort

Families with DCM were identified at 41 clinical centres in 12 countries across North America, Europe, Australia, and South Korea over a 30-year period (1993–2023). Probands and relatives were clinically assessed by expert teams at their referring centre, with medical history, physical examination, and cardiac investigations. Probands were diagnosed with DCM if there was left ventricular (LV) or biventricular systolic dysfunction and dilatation that was unexplained solely by abnormal loading conditions or coronary artery disease, in accordance with standard clinical practice.[10,11] DCM was considered familial if: (i) one or more first- or second-degree relatives had DCM, or (ii) there was an otherwise unexplained sudden cardiac death in a relative at any age with an established diagnosis of DCM.[10,11]

Genetic testing in probands was performed from 2012 onwards subsequent to the availability of next-generation sequencing techniques (multigene panels, exome or genome sequencing) to evaluate suites of cardiomyopathy-associated genes (typically 40–120 genes, including the 19 ClinGen-curated high- and moderate-evidence DCM disease genes).[12] Additional investigations to identify large copy number variants, such as comparative genomic hybridization arrays, were not routinely performed. Variant pathogenicity was classified in accordance with the American College of Medical Genetics and Genomics (ACMG) clinical guidelines and subsequent working group updates tailored for DCM.[13–17] For *TTN*tv, we also used a modified classification that incorporated the original ACMG criteria and recent ACMG recommendations for reporting secondary findings in *TTN* identified in clinical exome and genome sequencing.[13,16,18] Cascade testing of selected variants was performed in family members.

Families were included in this study if (i) a suspected disease-causing *TTN*tv had been identified (see details below), and (ii) genetic testing had been offered to affected and unaffected relatives. For the purposes of this study, family members were classified as affected if they had evidence of LV ejection fraction <50% with/without LV dilatation on transthoracic echocardiography or cardiac magnetic resonance imaging. All participants provided written informed consent for clinical data collection, genetic testing, and sharing of coded data for research purposes. Study protocols were approved by the St Vincent's Hospital and relevant institutional Human Research Ethics Committees.

Variant evaluation

TTN variants were annotated according to the inferred complete metatranscript (NM_001267550.2). Prioritized truncating variants were nonsense variants, small frame-shifting insertions or deletions that predictably lead to a stop codon, and splice-altering variants at canonical splice donor or acceptor sites (+/− 1–2 nucleotides at 5′ and 3′ positions of introns, respectively). Non-canonical splice variants included intronic variants outside the canonical +/− 1–2 positions, coding variants located at the end or start of an exon and missense variants within exons that were predicted to have a splicing impact. Exon usage across titin isoforms was assessed using percent spliced-in (PSI) scores as described[3] and data for normal adult ventricular tissue obtained from the Genotype-Tissue Expression (GTEx) portal. Variants were included if they were located in: (i) any exon with PSI score > .90 [N = 999 (95.5% of all *TTN*tv evaluated)], (ii) A-band or I-band exon with PSI score .89–.85 [N = 44 (4.2%)], or (iii) A-band or I-band exon with PSI score .84–.75 *and* supportive evidence of DCM association, e.g. family segregation or presence in multiple families [N = 3 (.3%)]. Included variants had a minor allele frequency of less than .0005 in a reference population database (gnomAD v4.0, accessed December 2023). Allele frequencies above this threshold level have been used to define benign variants.[14] Previously reported cases and variants are denoted in Supplementary data online, *Table S1*.

Variant splicing analysis

Variants in canonical and non-canonical splice sites were assessed using SpliceAI, a commonly used bioinformatics tool to predict splice-altering effects[19] and SpliceVault, a newly-developed program that predicts the most likely outcomes of splice-site changes based on observed events in human RNA-sequencing databases.[20] Expected outcomes were expressed as: out-of-frame (frameshift), in-frame, mixed (both out-of-frame and in-frame events likely), or no effect. Functional evaluation (mini-gene assay or RNA evaluation) to confirm these predictions was available for selected variants. Splice-site variants were annotated in accordance with recently published guidelines from the ClinGen Sequence Variant Interpretation Splicing Subgroup.[17] Further details about methods used to assess *TTN* splice-site variants are provided in the Supplementary Material.

*TTN*tv distribution analysis

The distribution and frequency of *TTN*tv across the various titin domains were evaluated in DCM patients and in a reference population database (gnomAD v4.0, accessed December 2023). Given the predominant European ancestry of our cohort, comparisons were made with data for the subset of non-Finnish Europeans within gnomAD. Variant locations were defined as follows:[21,22] Z-disk (start of exon 1 to the start of Ig-10 within exon 28), proximal I-band (Ig-10 within exon 28 to the end of exon 47), N2B unique sequence (exon 48), mid I-band (start of exon 49 to the end of exon 224), distal I-band (start of exon 225 to the end of Ig-107 within exon 250), A-band (Ig-108 within exon 251 to the end of the fibronectin 3–132 domain within exon 358), titin kinase (TK) domain (amino acids 33 813 to 34 136 within exon 358), and M-band (remainder of exon 358 following the TK domain to the end of exon 363). The number of *TTN*tv identified in each region was tallied and compared in the DCM and gnomAD control groups to calculate odds ratios (ORs) using GraphPad Prism.

Clinical risk factors

Clinical and genotype data were collected by collaborating centres. Core information provided to our study for all participants included sex, proband vs family member status, ethnicity, age at study entry, age at DCM diagnosis, age at last echocardiogram, and *TTN* genotype. Additional clinical information was available for a subset of participants. This included cardiac history, cardiac investigations (echocardiogram and/or cardiac magnetic resonance imaging, ECG), medications (cardiovascular, anthracycline chemotherapy), comorbidities (obesity, hypertension, ischaemic heart disease, diabetes, thyroid disease, chronic pulmonary disease), and lifestyle factors (alcohol consumption, physical activity levels, pregnancy) prior to or at the time of DCM diagnosis. Average weekly alcohol consumption was expressed as g/week, with heavy alcohol consumption defined as >140 g/week for males and >70 g/week for females.[23] Physical activity levels were categorized into high, moderate, or low. High physical activity was considered as ≥2.5 h of high activity (running, cycling, swimming, aerobics, organized sports, tasks that involve digging or lifting and carrying heavy loads, etc.) or >5 h of moderate activity (walking briskly, slow dancing, golf, social tennis, mowing the lawn, vacuuming or sweeping the floor, washing windows, etc.) per week. Moderate physical activity was defined as ≥2.5 h of moderate or <2.5 h

Table 1 Demographic data used for analysis of penetrance in the primary cohort

Parameter	All subjects (*N* = 3106)	Probands[a] (*N* = 966)	Relatives (*N* = 2140)
Proband (%)	966 (31.1%)[a]	966 (100%)	0 (0%)
Male (%)	1711 (55.1%)	664 (68.7%)	1047 (48.9%)
DCM (%)	1625 (52.3%)	920 (95.2%)	705 (32.9%)
Age at DCM (yr; median, IQR)	48 (.11–90)	47 (.11–81)	50 (7–90)
Age at study (yr; median, IQR)	52 (.11–95)	56 (.11–90)	50 (3–95)
Ancestry (%):			
– European	2991 (96.3%)	915 (94.7%)	2076 (97%)
– Other	115 (3.7%)	51 (5.3%)	64 (3%)
*TTN*tv-positive (% individuals)	2388 (76.9%)	966 (100%)	1422 (66.5%)
*TTN*tv type (% variants):			
– Nonsense	1058 (44.3%)	415 (43%)	643 (45.2%)
– Frameshift	1029 (43.1%)	441 (45.7%)	588 (41.4%)
– Splice (canonical site)	301 (12.6%)	110 (11.4%)	191 (13.4%)
*TTN*tv location (% variants):			
– Z-disk	65 (2.7%)	27 (2.8%)	38 (2.7%)
– I-band	420 (17.6%)	164 (17%)	256 (18%)
– A-band	1824 (76.4%)	740 (76.6%)	1084 (76.2%)
– M-band	79 (3.3%)	35 (3.6%)	44 (3.1%)

[a]Proband information was only available for 966 of the 1043 families in the study. Probands in the remaining 77 families were seen outside of the centres involved in the current study and data were unavailable for analysis.

of high activity per week. Low physical activity was defined as <2.5 h of moderate activity per week.[24]

Statistical analysis

Characteristics of study participants are presented as *N* (%) for categorical data, median [interquartile range (IQR)] for non-normally distributed continuous data, or mean [standard deviation (SD)] for normally distributed continuous data. For analyses of DCM penetrance in *TTN*tv-positive and *TTN*tv-negative individuals, we used age at DCM onset for any individual diagnosed with DCM (phenotype-positive), and censored individuals without DCM (phenotype-negative) at their age at the last echocardiogram (for *TTN*tv-positive) or age at study entry (for *TTN*tv-negative). Differences in the lifetime risk of DCM in *TTN*tv-positive and *TTN*tv-negative individuals were assessed using shared frailty survival models adjusted for sex and with family relatedness included as a random effect to account for intra-cluster correlation, then visualized with cumulative incidence plots generated with the Kaplan-Meier method. Among *TTN*tv-positive individuals, we further evaluated differences in DCM penetrance by proband vs family member status, sex, variant type (nonsense, frameshift insertion/deletion, splice-altering), type of splice site (canonical, non-canonical), splice consequence (out-of-frame, in-frame, mixed, no effects), and titin region (N2B unique sequence/distal I-band/A-band, vs. other band regions).

We conducted analyses of associations of risk factors with DCM in a subset of the cohort with available information on clinical risk factors, and with known information on sex and ethnicity. We imputed missing data using multiple imputation by chained equations (known as MICE) with 10 imputed datasets and used multivariate mixed-effects logistic regression to estimate ORs and corresponding 95% confidence intervals (CIs) for key risk factors with DCM. Models were mutually adjusted for age, sex, ethnicity, past or current heavy alcohol use, exercise intensity, body mass index (BMI), history of hypertension, history of ischaemic heart disease, history of diabetes, history of pulmonary disease, history of thyroid disease, history of atrial fibrillation (AF), and medication use prior to DCM diagnosis, i.e. beta-adrenergic receptor and/or renin-angiotensin system (RAS) blocking drugs (including angiotensin-converting enzyme inhibitors and angiotensin receptor blockers) as fixed effects, and family relatedness was included as a random effect. We further evaluated associations of any risk factor (including any DCM-promoting risk factor or heart failure comorbidity), any known DCM-promoting risk factor (AF, heavy alcohol intake, BMI >35 kg/m^2, pregnancy, anthracycline chemotherapy),[25,26] any heart failure-associated comorbidity (diabetes, hypertension, ischaemic heart disease, pulmonary disease, and thyroid disease).[26,27] We also evaluated associations of DCM-protective factors (beta-adrenergic receptor and/or RAS blockers, moderate or high levels of exercise) with DCM. Analyses were conducted in the study cohort overall and by sex and age group (<30 years, 30 to <60 years, ≥60 years). Sensitivity analyses were undertaken using individuals with complete data. Statistical tests were two-sided, and a *P*-value $< .05$ was considered statistically significant. All analyses were performed using R (version 4.3.2). The R libraries used for multiple imputation, shared frailty survival models, and mixed-effects logistic regression models were *mice* (version 3.17.0), *coxme* (version 2.2–22), and *lme4* (version 1.1–37), respectively.

Results

DCM penetrance

A total of 3158 subjects in 1043 families with *TTN*tv-related DCM were studied (see Supplementary data online, *Figure S1*). Of these, 3106 subjects in families with clinically reportable *TTN*tv (nonsense, frameshift,

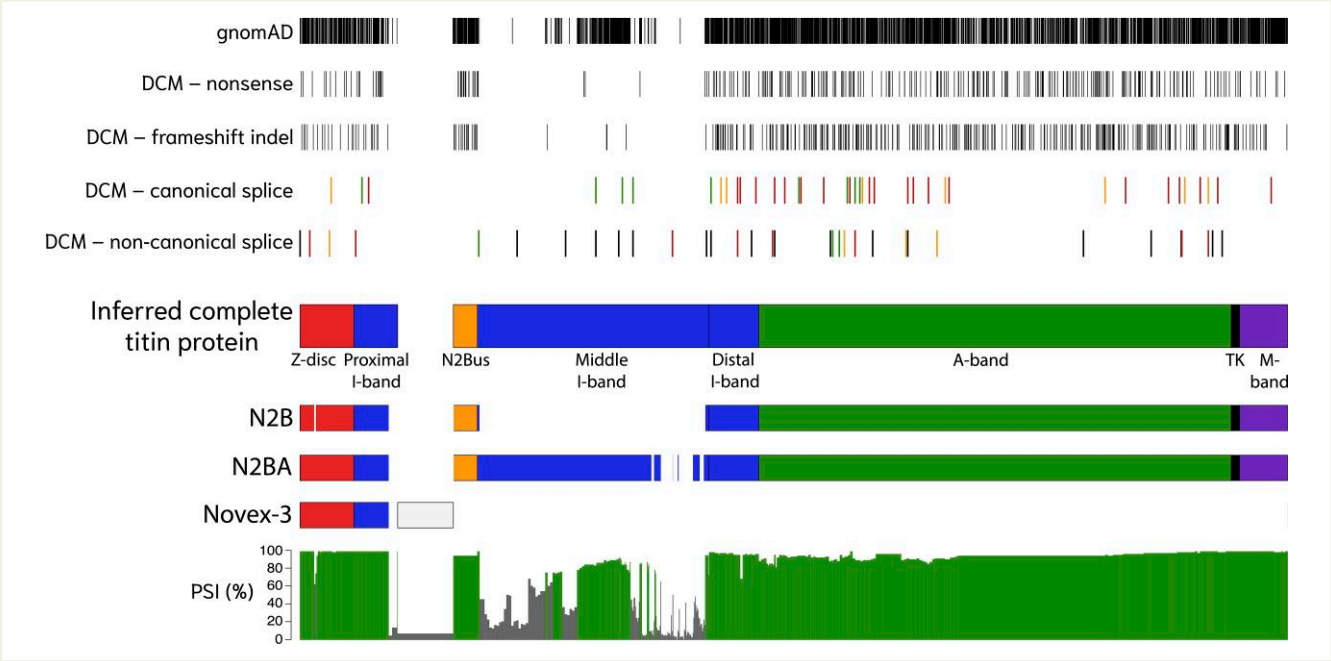


Figure 1 Distribution of DCM-associated *TTN*tv. *TTN*tv identified in families with DCM are plotted with respect to the gnomAD population database and their location in the inferred complete titin protein (meta-transcript NM_001267550.2): Z-disk (red), I-band (blue), A-band (green) and M-band (purple). *TTN*tv are denoted by vertical bars; consequences of splice-site variants are shown: frameshift (red), in-frame (green), mixed effects (orange). Differences in exon usage across the various titin isoforms are indicated by percent spliced-in (PSI) scores. *TTN*tv in exons with PSI scores >.75 (green) were included in this study

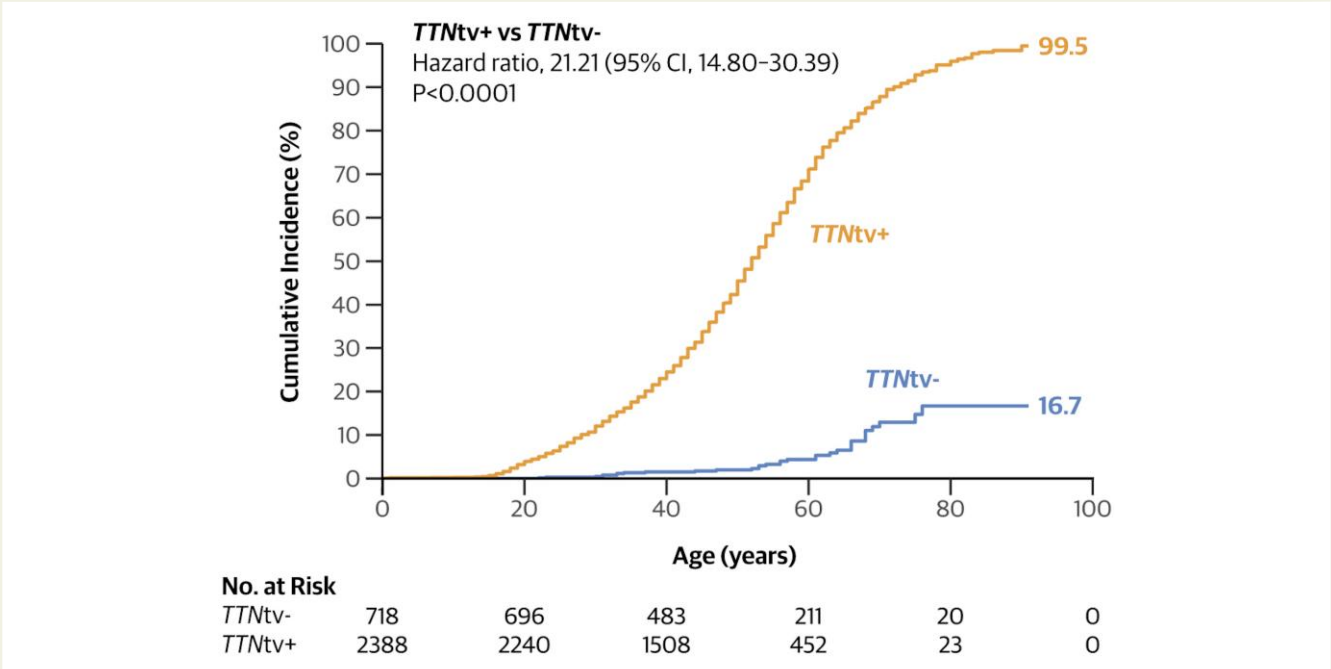


Figure 2 Association of *TTN*tv with DCM. Curves show the cumulative incidence of DCM over time adjusted for sex and family relatedness in genotype-positive (*TTN*tv+) and genotype-negative (*TTN*tv-) subjects

or canonical splice-site change) comprised the primary cohort. The remaining 52 subjects with non-canonical splice-site changes were evaluated as a separate cohort.

In the primary cohort, there were 966 *TTN*tv-positive probands (69% males) and 2140 relatives (49% males) of whom 1422 (66%) were *TTN*tv-positive and 718 (34%) were *TTN*tv-negative (*Table 1*).

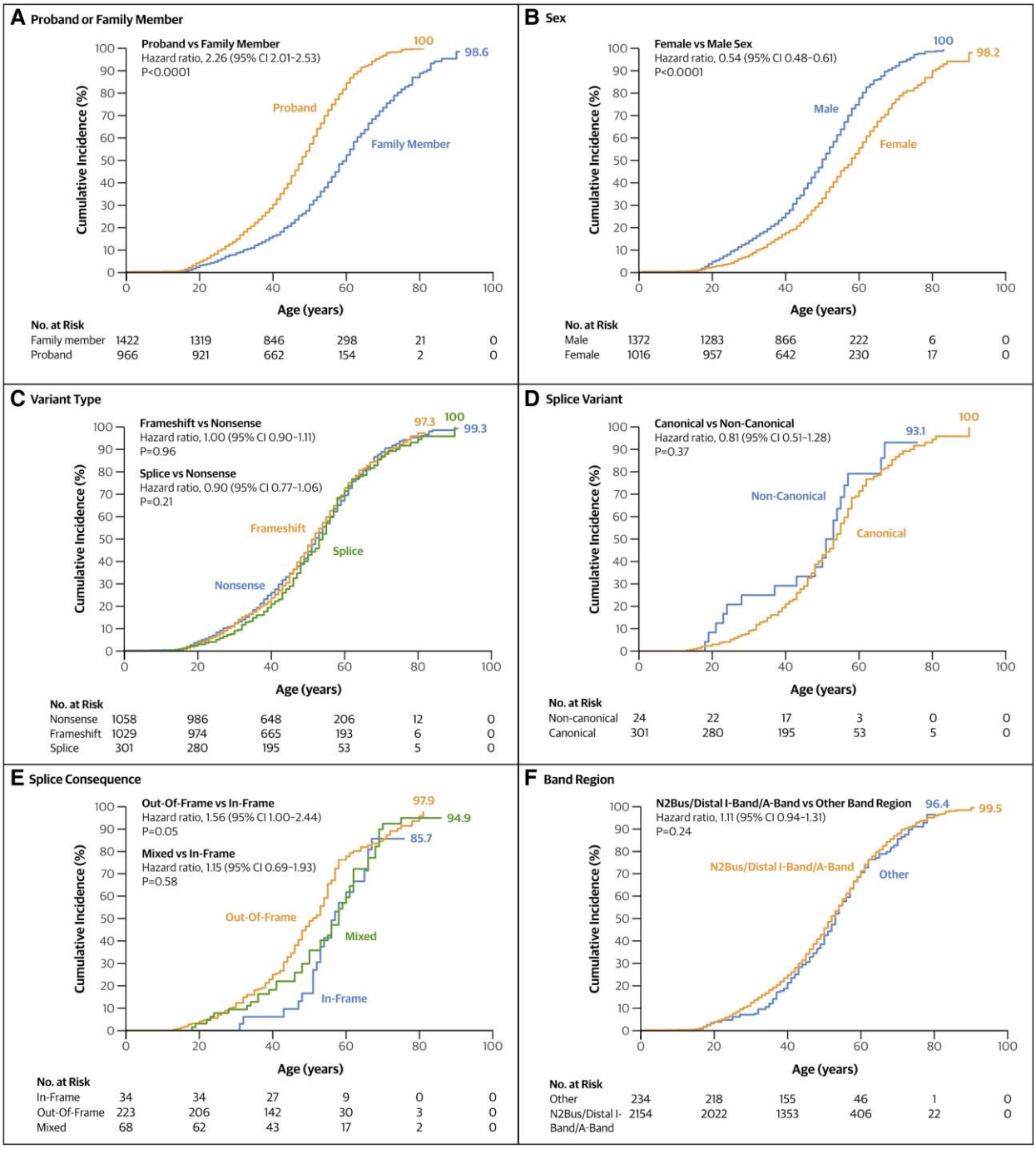


Figure 3 Associations of factors with DCM penetrance in *TTN*tv-positive subjects. Survival curves show associations of proband vs family member status (panel *A*), sex (panel *B*), and variant characteristics (panels *C–F*). The latter includes the three main types of *TTN*tv (nonsense, frameshift insertion/deletion, and canonical splice-site change; panel *C*), a comparison of splice-altering variants in canonical and non-canonical sites (panel *D*), predicted downstream consequences of splice-altering variants (out-of-frame, in-frame, mixed effects; panel *E*), and titin domain location (panel *F*)

There were 705 *TTN*tv (294 nonsense variants, 364 frameshift insertions or deletions, and 47 variants in canonical splice sites) in 1043 families; 540 variants were unique to single kindreds and 165 were recurrent in 2–20 families, potentially representing founder variants (*Figure 1*, Supplementary data online, *Table S1*). The majority (98.6% of primary cohort) of *TTN*tv-positive subjects were heterozygous for a single *TTN*tv; three subjects (.1% of primary cohort; all with DCM) had a second prioritized *TTN*tv (compound heterozygous, $N = 1$; phase unknown, $N = 2$). Rare pathogenic or likely pathogenic variants in another DCM gene were present in 30 *TTN*tv-positive subjects (1.3% of the primary cohort; 27 with DCM) and 5 *TTN*tv-negative subjects all without DCM (see Supplementary data online, *Table S2*).

Table 2 Sex-specific probabilities and corresponding 95% confidence intervals for developing DCM at each decade of age in *TTN*tv-positive (*TTN*tv+) and *TTN*tv-negative (*TTN*tv−) subjects

Age (years)	Males			Females			All subjects (*N* = 3106)
	*TTN*tv+ (*N* = 1372)	*TTN*tv− (*N* = 339)	Total (*N* = 1711)	*TTN*tv+ (*N* = 1016)	*TTN*tv− (*N* = 379)	Total (*N* = 1395)	
10	.1 (.0–.2)	.0 (.0–.0)	.1 (.0–.2)	.5 (.1–.9)	.0 (.0–.0)	.4 (.0–.7)	.2 (.0–.3)
20	5.0 (3.8–6.1)	.0 (.0–.0)	4.0 (3.1–4.9)	2.4 (1.5–3.4)	.0 (.0–.0)	1.8 (1.1–2.4)	3.0 (2.4–3.6)
30	14.8 (12.9–16.7)	.6 (.0–1.5)	12.1 (10.5–13.6)	8.3 (6.5–10.0)	.3 (.0–.9)	6.1 (4.8–7.4)	9.4 (8.4–10.5)
40	28.4 (25.930.9)	2.5 (.6–4.3)	23.7 (21.5–25.8)	18.9 (16.3–21.5)	.7 (.0–1.6)	14.2 (12.2–16.2)	19.5 (18.0–21.0)
50	52.5 (49.6–55.3)	2.5 (.6–4.3)	44.3 (41.6–46.8)	34.8 (31.4–38.1)	1.6 (.0–3.1)	26.7 (23.9–29.3)	36.8 (34.8–38.7)
60	79.1 (76.4–81.4)	3.8 (1.2–6.4)	68.8 (66.0–71.3)	58.0 (54.0–61.7)	5.0 (1.6–8.2)	46.6 (43.0–49.9)	59.7 (57.5–61.9)
70	92.8 (90.9–94.4)	11.8 (4.8–18.3)	84.1 (81.5–86.4)	78.9 (74.9–82.3)	14.0 (6.0–21.3)	67.1 (62.9–70.8)	77.3 (75.0–79.4)
80	98.6 (97.3–99.3)	11.8 (4.8–18.3)	92.4 (89.7–94.4)	90.9 (86.4–93.9)	21.2 (8.4–32.2)	81.2 (75.6–85.5)	88.0 (85.4–90.1)
90	100	11.8 (4.8–18.3)	95.9 (91.3–98.1)	98.2 (89.9–99.7)	21.2 (8.4–32.2)	93.0 (79.8–97.6)	95.2 (89.3–97.9)

Table 3 Distribution of DCM-associated *TTN*tv in different titin regions

Titin region[a]	*TTN*tv+ probands		Prevalence		Odds ratio			*P*-value
	DCM cohort (*N* = 1043 subjects)	Controls[b] (*N* = 590 031 subjects)	DCM cohort (%)	Controls (%)	Mean	Max	Min	
Z-disk	30	254	2.88	.04	68.8	100.9	46.9	<.001
I-band: Proximal	31	258	2.97	.04	70.0	102.2	48.0	<.001
I-band: N2Bus	71	141	6.81	.02	305.6	409.3	228.2	<.001
I-band: Mid	18	1496	1.73	.25	6.9	11.0	4.3	<.001
I-band: Distal	62	250	5.94	.04	149.1	198.3	112.1	<.001
A-band: Non-TK	782	1888	74.98	.32	933.4	1081.0	805.6	<.001
A-band: TK	13	39	1.25	.01	190.9	358.7	101.6	<.001
M-band	39	469	3.74	.08	48.8	68.1	35.0	<.001

N2Bus, N2B unique sequence; TK, titin kinase domain; *TTN*tv+, *TTN*tv-positive.
[a]All *TTN*tv evaluated were located in constitutively expressed exons in normal adult human heart. Domain boundaries were sourced from TITINdb.[21,22]
[b]Control data were derived from non-Finnish Europeans in the gnomAD (v.4.0) population database.

Additionally, nine subjects (six with DCM) from three families had a prioritized *TTN*tv and a non-canonical *TTN* splice variant or a missense *TTN* variant with proven functional impact.

*TTN*tv-positive subjects were 21-fold more likely to develop DCM than *TTN*tv-negative subjects (OR, 21.21; 95% CI; 14.80–30.39) (*Figure 2*, Supplementary data online, *Table S3*). Disease onset was earlier in *TTN*tv-positive probands when compared to family members (*Figure 3A*) and in males compared to females (*Figure 3B*); 88% *TTN*tv-positive subjects (93% males, 79% females) developed DCM by age 70 years (*Table 2*, Supplementary data online, *Table S3*). The mean age at DCM onset in subjects with a single *TTN*tv (47 ± 15 years) was not significantly different from that in subjects with a second *TTN*tv or rare variant (44 ± 19 years; $P = .69$). Data for DCM penetrance were similar when these individuals were excluded (see Supplementary data online, *Table S4*).

Associations of variant type and location

There were no significant differences in the penetrance of DCM between subjects with the three main types of *TTN*tv, i.e. nonsense, frameshift insertions/deletions, and canonical splice-site changes (*Figure 3C*). Splice-altering variants can result in exon skipping, activation of cryptic donor/acceptor sites, or intron retention, with a variety of downstream outcomes that can be difficult to predict without RNA analysis. Using SpliceVault,[20] we grouped *TTN* splice-site variants based on expected out-of-frame (frameshift), in-frame, mixed, or no effects. When variants with positive effects were considered, there were no differences in DCM penetrance associated with different splice-site locations (canonical vs non-canonical; *Figure 3D*, Supplementary data online, *Tables S1* and *S5*) or consequences (out-of-frame vs. in-frame vs. mixed effects; *Figure 3E*, Supplementary data online, *Table S6*).

Table 4 Baseline characteristics of *TTN*tv-positive subjects included in the Risk Factor analysis

	All subjects (*N* = 1441)	DCM present (*N* = 931)	DCM absent (*N* = 510)
Proband (%)	537 (37.3%)	503 (54%)	34 (6.7%)[a]
Male (%)	811 (56.3%)	596 (64%)	215 (42.2%)
DCM (%)	931 (64.6%)	931 (100%)	0 (0%)
Age at DCM diagnosis (yr; median, IQR)	49 (.4–90)	49 (.4–90)	NA
Age at study (yr; median, IQR)	53 (3–91)	58 (5–91)	42 (3–87)
Ancestry (%):			
– European	1360 (94.4%)	874 (93.9%)	486 (95.3%)
– Other	81 (5.6%)	57 (6.1%)	24 (4.7%)
Alcohol excess	134/1042 (12.9%)	108/681 (15.9%)	26/361 (7.2%)
Any AF (%)	320/1437 (22.2%)	276/927 (29.8%)	44/510 (8.6%)
AF at or prior to DCM diagnosis (%)	192/1437 (13.4%)	148/927 (16.0%)	44/510 (8.6%)
BMI >35 kg/m^2 (%)	100/1246 (8%)	69/817 (8.5%)	31/429 (7.2%)
Chemotherapy (%)	11/1405 (.8%)	10/903 (1.1%)	1/502 (.2%)
Pregnancy (%)[b]	29/620 (4.7%)	29/330 (8.8%)	0/290 (0%)
Hypertension (%)	248/1378 (18%)	166/878 (18.9%)	82/500 (16.4%)
Ischaemic heart disease (%)	65/1391 (4.7%)	51/889 (5.7%)	14/502 (2.8%)
Diabetes (%)	103/1397 (7.4%)	81/892 (9.1%)	22/505 (4.4%)
Thyroid disease (%)	97/1408 (6.9%)	68/904 (7.5%)	29/504 (5.8%)
Chronic lung disease (%)	66/1238 (5.3%)	49/799 (6.1%)	17/439 (3.9%)
BB/RAS use prior to DCM diagnosis (%)	161/1332 (12.1%)	66/837 (7.9%)	95/495 (19.2%)
Exercise (mod/high) (%)	518/1021 (50.7%)	307/649 (47.3%)	211/372 (56.7%)
LVAD (%)	50 (3.5%)	50 (5.4%)	0 (0%)
Heart transplant (%)	97 (6.7%)	97 (10.4%)	0 (0%)
NSVT/VT/VF (%)	294 (20.4%)	278 (29.9%)	16 (3.1%)
ICD/CRT (%)	281 (19.5%)	271 (29.1%)	10 (2%)
Aborted cardiac arrest (%)	41 (2.8%)	36 (3.9%)	5 (1%)
Sudden cardiac death (%)	19 (1.3%)	16 (1.7%)	3 (.6%)
Conduction disease[c] (%)	151 (10.5%)	138 (14.8%)	13 (2.5%)
PPM[d] (%)	13 (.9%)	12 (1.3%)	1 (.2%)

AF, atrial fibrillation; BB/RAS beta-adrenergic receptor and/or renin-angiotensin system-blocking drug therapy; BMI, body mass index; CRT, cardiac resynchronization therapy; ICD, implantable cardioverter defibrillator; LVAD, left ventricular assist device; NA, not applicable; NSVT, non-sustained ventricular tachycardia; PPM, permanent pacemaker; VF, ventricular fibrillation; VT, ventricular tachycardia.
[a]Identified as a proband in a family with DCM following resuscitated cardiac arrest, AF diagnosis, or LV dilation with EF > 50%.
[b]Denominator numbers include only female participants.
[c]Conduction disease included first/second/third-degree atrioventricular block or left/right bundle branch block.
[d]Seven of thirteen individuals also received an ICD or CRT subsequent to PPM implantation.

As reported previously,[2,3] the majority of disease-associated *TTN*tv were situated in the titin A-band (excluding TK domain) (*Figure 1*, *Table 3*). Comparing the distribution of *TTN*tv in our DCM cohort with a reference control population revealed additional clusters in the I-band exon 48, which encodes the N2B unique sequence, and the distal A-band TK domain (*Table 3*).[21,22] DCM penetrance was similar for *TTN*tv in constitutively expressed exons across all titin domains (*Figure 3F*). Adhering to the widely used 2020 guidelines for clinical interpretation of *TTN*tv in DCM patients that prioritizes A-band variants,[14] only 40% of our variants would be classified as pathogenic/likely pathogenic. The yield of pathogenic/likely pathogenic variants rises to 99% if the evidence level for a truncating variant is increased to very strong or strong[13,16] and *TTN*tv in all high PSI exons (A-band and non-A-band)[18] are included (see Supplementary data online, *Table S1*).

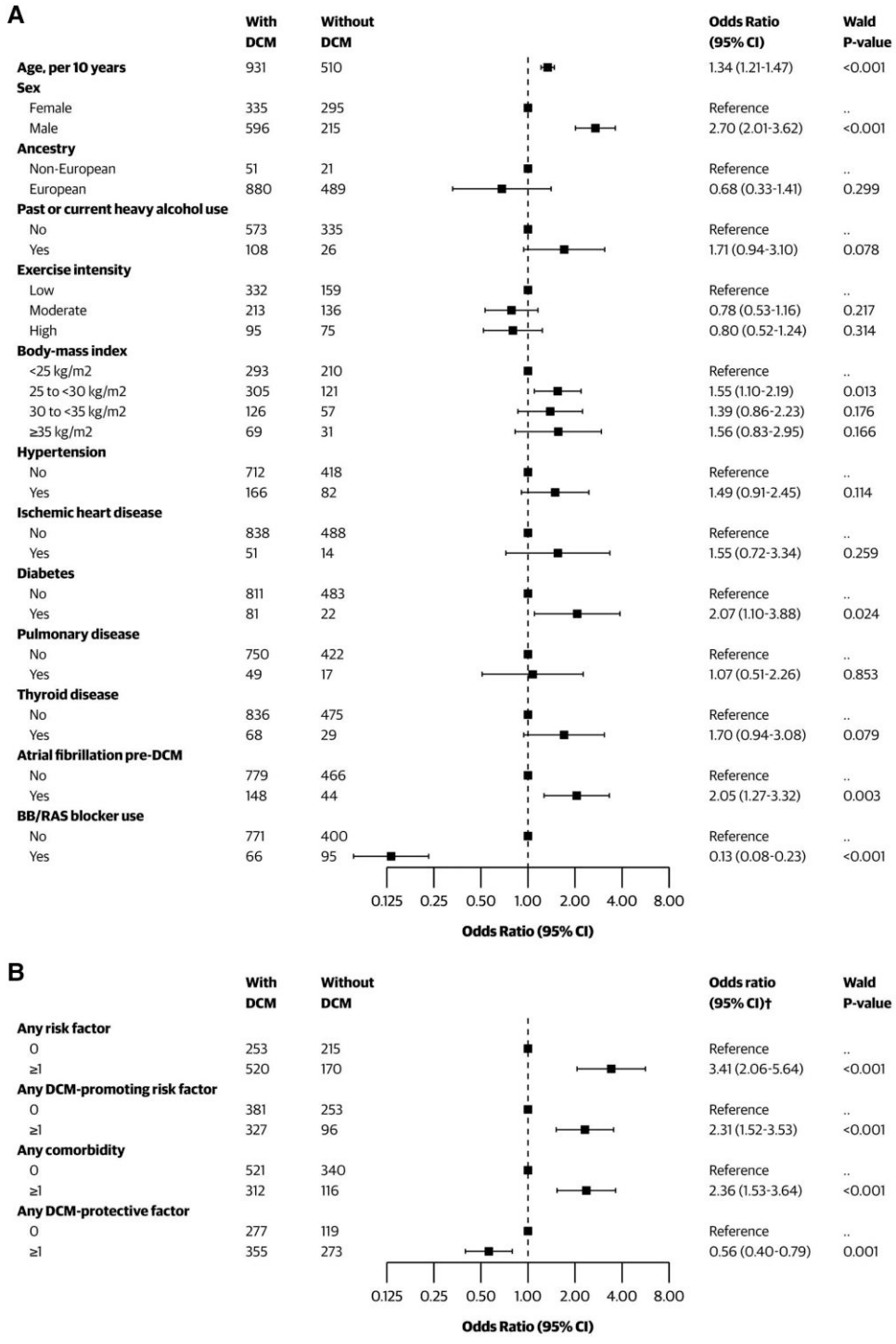


Figure 4 Associations of clinical risk factors with DCM penetrance in *TTN*tv-positive subjects. Forest plots show odds ratios and corresponding 95% confidence intervals (CI) for DCM associated with single risk factors (panel *A*), or combinations of risk factors (panel *B*). See text for definitions of risk factor combinations. Odds ratios and 95% CIs were estimated across ten imputed datasets generated using multiple imputations by chained equations and pooled using Rubin's Rules. BB/RAS, beta-adrenergic receptor or renin-angiotensin system-blocking drugs

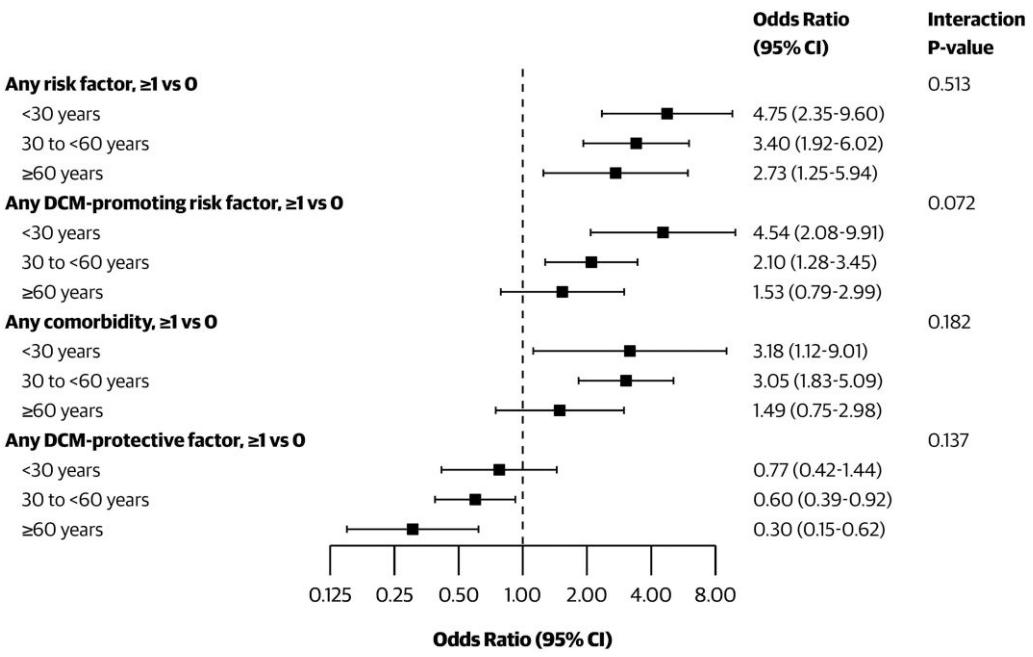


Figure 5 Comparison of risk factor prevalence in different age groups. Forest plot shows odds ratios and corresponding 95% confidence intervals of risk factor combinations with DCM according to age at DCM diagnosis (<30 years, 30 to <60 years, ≥60 years). Odds ratios and 95% CIs were estimated across ten imputed datasets generated using multiple imputations by chained equations and pooled using Rubin's Rules

Clinical factors associated with earlier DCM onset

Information about comorbidities and lifestyle patterns was available for a subset of 1441 probands and relatives, of whom 931 (65%) had DCM (*Table 4*). There were strong associations of DCM with age, male sex, and the presence of cardiovascular risk factors prior to DCM diagnosis (*Figure 4*). The latter comprised factors that have been independently considered DCM-promoting, including heavy alcohol intake, class II/III obesity (BMI >35 kg/m^2), pregnancy, anthracycline chemotherapy,[25,26] as well as factors associated more broadly with heart failure but not specifically with DCM, including diabetes, hypertension, ischaemic heart disease, pulmonary disease, and thyroid disease (*Figure 4B*).[26,27] These risk factor associations were seen in males and females (see Supplementary data online, *Figure S2*).

AF was documented in 320/1441 *TTN*tv-positive subjects (22%; Supplementary data online, *Table S7*). In 120/320 subjects (37%), AF was diagnosed after DCM. However, in 192/320 subjects (60%), AF was diagnosed either prior to DCM ($n = 72$), coincident with DCM ($n = 76$), or in the absence of DCM ($n = 44$). The majority of these 192 subjects (64%) had established AF-promoting clinical risk factors in addition to carrying a *TTN*tv, including hypertension, heavy alcohol intake, diabetes, and sedentary lifestyle.[28] Having a history of AF was associated with a two-fold increased odds of DCM development (OR, 2.05; 1.27–3.32; *Figure 4A*).

Clinical factors associated with later DCM onset

Factors that protect against disease onset in familial DCM have not previously been identified. We observed a marked reduction (87%) in the odds of DCM (OR .13, 95% CI .08–.23) in *TTN*tv-positive subjects who had been receiving beta-adrenergic receptor or RAS-blocking drug therapy prior to their DCM diagnosis (*Figure 4A*). These drugs had been typically commenced for indications such as hypertension, AF, symptomatic ventricular ectopy, or ischaemic heart disease with estimated treatment duration that preceded DCM by many years (median, 84 months; range, 1–492 months). There was a trend for regular moderate/high levels of exercise to have beneficial effects when compared to a sedentary lifestyle, especially for females (*Figures 4A*, Supplementary data online, *Figure S2A*).

Distinctive risk factor profiles in the young and old

Clinical risk factor patterns differed in subjects with young-onset (<30 years) and older-onset (≥60 years) DCM (*Figure 5*, *Table 5*, Supplementary data online, *Table S8*). In the young-onset cases, DCM was diagnosed following a symptomatic presentation or as a result of family echocardiographic screening. The most frequently identified triggers were acute infection and atrial or ventricular tachyarrhythmias. In young males, the most common risk factors were heavy alcohol intake (40%), AF (28%), and class II/III obesity (20%) (*Table 5*). In young females, the single most prevalent factor was pregnancy (58%), followed by class II/III obesity (25%) and thyroid disease (21%). Hypertension and AF were the top two risk factors in males and females diagnosed with DCM ≥60 years. Nineteen percent of young-onset cases and 45% of older-onset cases had multiple risk factors.

The prevalence of clinical risk factors increased with age, ranging from 28% in those with young-onset DCM to 68% in late-onset DCM. These risk factors were strongly associated with DCM in all age groups; however, the greatest odds of DCM were seen in the young-onset cases (OR, 4.75; 95% CI; 2.35–9.60; *Figure 5*). This was most notable for class II/III obesity, diabetes, and thyroid disease (see

Table 5 Different risk factor profiles in *TTN*tv-positive males and females with early- and late-onset DCM

DCM diagnosis <30 years			DCM diagnosis ≥60 years		
***DCM-exacerbating factors*[a]**					
Males (*N* = 50)	**Females (*N* = 24)**	**All (*N* = 74)**	**Males (*N* = 103)**	**Females (*N* = 53)**	**All (*N* = 156)**
Alcohol (40.0%)	Pregnancy (58.3%)	Alcohol (28.4%)	AF (47.6%)	Hypertension (52.8%)	Hypertension (41.7%)
AF (28.0%)	BMI > 35 (25.0%)	AF (21.6%)	Hypertension (35.9%)	AF (26.4%)	AF (40.4%)
BMI > 35 (20.0%)	Thyroid (20.8%)	BMI > 35 (21.6%)	Ischaemic HD (24.3%)	Chronic lung (18.9%)	Ischaemic HD (19.9%)
Diabetes (14.0%)	AF (8.3%)	Pregnancy (18.9%)	Alcohol (17.5%)	Diabetes (15.1%)	Diabetes (16.0%)
Hypertension (12.0%)	Alcohol (4.2%)	Thyroid (12.2%)	Diabetes (16.5%)	Thyroid (15.1%)	Alcohol (14.1%)
Thyroid (8.0%)	Diabetes (4.2%)	Diabetes (10.8%)	Chronic lung (9.7%)	Ischaemic HD (11.3%)	Chronic lung (12.8%)
ChemoRx (6.0%)		Hypertension (8.1%)	Thyroid (7.8%)	Alcohol (7.5%)	Thyroid (10.3%)
Chronic lung (4.0%)		ChemoRx (4.1%)	BMI > 35 (4.9%)	BMI > 35 (1.9%)	BMI > 35 (3.8%)
Ischaemic HD (2.0%)		Chronic lung (2.7%)		ChemoRx (1.9%)	ChemoRx (.6%)
		Ischaemic HD (1.4%)			
DCM-protective factors					
Males (N = 73)	**Females (N = 16)**	**All (N = 89)**	**Males (N = 53)**	**Females (N = 30)**	**All (N = 83)**
Exercise (91.8%)[b]	Exercise (93.7%)[b]	Exercise (92.1%)[b]	Exercise (58.5%)[b]	BB/RAS Rx (46.7%)	Exercise (53.0%)[b]
BB/RAS (5.5%)	Both (6.3%)	BB/RAS (4.5%)	BB/RAS (26.4%)	Exercise (43.3%)[b]	BB/RAS (33.7%)
Both (2.7%)		Both (3.4%)	Both (15.1%)	Both (10.0%)	Both (13.3%)

AF, atrial fibrillation; BB/RAS beta-adrenergic receptor and/or renin-angiotensin system-blocking drug therapy prior to DCM diagnosis; BMI, body mass index; chemoRx, anthracycline chemotherapy; HD, heart disease.
[a]14/74 (18.9%) of the <30 years group presented with multiple risk factors (11 males and 3 females) and 70/156 (44.9%) of the ≥60 years group presented with multiple risk factors (48 males and 22 females).
[b]Moderate or high levels of exercise.

Supplementary data online, *Figure S3*). The negative association of pharmacological therapy with DCM was relatively less in subjects with young-onset DCM, which most likely reflects the lower frequency and shorter duration of treatment. For all the various risk factor associations, sensitivity analyses of individuals with complete data were consistent with the primary findings (see Supplementary data online, *Figures S4–S7*).

Discussion

Here, we show that *TTN*tv-positive members of families with DCM can expect to develop disease during their lifetime. However, the age of DCM onset is highly variable, ranging from childhood to late adult life. We evaluate factors that contribute to this variability and highlight the importance of the individual patient environment. Our findings expand the spectrum of potentially deleterious *TTN*tv and identify clinical factors associated with earlier or later DCM onset. Collectively, these findings reveal new opportunities for DCM prevention and improved family management (*Structured Graphical Abstract*).

Clinical reporting of *TTN*tv focuses on nonsense, frameshift insertions/deletions, and canonical splice-site variants in the titin A-band,[14] with variants of other types and locations often not reported or deemed to have uncertain significance due to insufficient evidence to support disease causation. This has likely led to considerable under-recognition of clinically relevant *TTN*tv. In our families, most DCM-associated variants occurred in the titin A-band with additional hotspots in the N2B unique sequence and TK domain, which have been implicated in sensing and responding to mechanical stress.[29,30] DCM-associated *TTN*tv in high PSI exons outside these regions were relatively less frequent but had similar DCM penetrance. These data argue for a review of DCM variant classification matrices with upgrading of *TTN*tv in any high PSI exon irrespective of location. Splice-altering variants in non-canonical sites or with in-frame effects also warrant further consideration. Inclusion of non-canonical splice-site variants has been proposed to increase the diagnostic yield of *TTN* sequencing by 10%–20%.[31] Here, we extend these findings by showing that these variants have similar DCM penetrance to that observed for canonical splice-site variants. Our data suggest that splice-site variants with in-frame effects can be associated with DCM. However, these results need to be interpreted with caution as functional consequences may vary with the size of the inserted or deleted segment.

Since *TTN*tv often occurs as incidental findings in the general population, it has been speculated that additional factors are required for disease manifestation. Experimental data in human cardiomyocyte and zebrafish models suggest that *TTN*tv are sufficient to cause DCM.[32,33] The individual patient context appears to be highly relevant, however, as a determinant of the timing of DCM onset. *TTN*tv-positive subjects are thought to be more susceptible to developing DCM in settings of haemodynamic stress (e.g. pregnancy)[34,35] or exogenous toxins (e.g. alcohol excess, anthracycline chemotherapy).[36,37] In our study, pregnancy was identified as a period of heightened DCM risk in young

females, while alcohol excess was a major factor in young males. Anthracycline chemotherapy exposure occurred mainly in young males and older females but accounted for only a small proportion of DCM cases.

Apart from age and male sex, AF had the strongest positive association with DCM in our families. AF is a common complication of severe DCM due to any cause and is likely related to atrial dilatation. Of note, *TTN*tv have been associated with early-onset and lone AF in the absence of discernible ventricular cardiomyopathy, raising the possibility of a primary atrial cardiomyopathy and arrhythmogenic substrate.[38–42] In keeping with this, two-thirds of our *TTN*tv-positive subjects who developed AF were diagnosed prior to or coincident with DCM. However, the majority of these subjects had both a *TTN*tv *and* one or more established clinical risk factors for AF. Further, we are unable to exclude the potential role of additional rare or common AF-promoting genetic variants. These findings suggest that the aetiology of *TTN*tv-associated AF can be complex, and careful assessment of contributing factors in each patient is needed. Irrespective of the cause, rapid AF can accelerate DCM progression or trigger acute decompensation and was treated as a DCM-promoting factor in our analyses.

Comorbidities such as obesity, hypertension, and diabetes, are major contributors to the global burden of cardiovascular disease, including heart failure, arrhythmias, and stroke,[43] but are not typically considered as DCM-promoting. Our data now suggest that overall cardiometabolic health influences disease onset in *TTN*tv-related DCM. As expected, the prevalence of comorbidities increased with age in our cohort. Alarmingly, however, the strongest associations with DCM were seen in young-onset cases. Obesity was of particular concern, with one in five individuals diagnosed with DCM under 30 years of age being classified as morbidly obese (class II/III). Obesity is most often associated with heart failure with preserved ejection fraction but can also promote progressive LV dilatation and DCM.[44,45]

The role of exercise is a contentious topic in genetic cardiomyopathies, with varying evidence for disease-accelerating vs beneficial effects according to exercise duration and intensity, cardiomyopathy type, symptom status, and underlying genotype.[10] In our study, there appeared to be an inverse correlation between exercise amount and DCM onset. This could indicate that low levels of exercise exacerbate DCM risk, especially since obesity and a sedentary lifestyle are frequently intertwined. Alternatively, moderate/high levels of exercise might be cardioprotective. A recent small study in patients with established *TTN*tv-related DCM showed that exercise training improved cardiovascular fitness,[46] favouring the latter hypothesis. While these data collectively support the concept of "exercise as therapy", the extent to which regular exercise might delay or prevent DCM onset remains to be determined. It should be noted that none of our study subjects were elite competitive endurance athletes, in whom impaired ventricular function is not uncommon.[47] Further studies are needed to more closely define safe and effective levels of exercise as well as mechanisms of cardioprotective effects in *TTN*tv-positive individuals.

A striking finding of our study was the reduced likelihood of DCM associated with use of beta-adrenergic receptor or RAS-blocking drug treatment prior to DCM diagnosis. To date, evidence-based guidelines for disease prevention in familial DCM have been lacking. Our data now provide a strong foundation for a randomized clinical trial to address this issue. We expect that pre-emptive therapy in *TTN*tv-positive subjects with subclinical cardiomyopathy (e.g. LV ejection fraction 50%–55% or impaired global longitudinal strain) would be beneficial.[48]

Several points need to be considered when interpreting our findings. The number of genotype-negative family members was less than the 50:50 ratio expected in an autosomal dominant disease. This is not an uncommon clinical scenario where there may be physician bias to undertake genetic testing in affected individuals or patient reluctance to be tested in the absence of symptoms. Despite this imbalance, there was a clear positive association of *TTN*tv with DCM status, in keeping with previous reports.[2,3] The relatively lower proportion of genotype-negative relatives did not influence any subsequent analyses as these were only performed in genotype-positive probands and relatives. The *TTN*tv evaluated here were all identified in kindreds with DCM and hence selected for disease association. Caution is needed in interpreting non-canonical *TTN* splice-site changes since variants identified in families may be subject to selection bias and predictions based on *in silico* tools alone may overestimate the yield of clinically-significant findings. Factors other than *TTN*tv *per se* may contribute to the high lifetime risk of DCM in *TTN*tv-positive family members, including shared genetic background or environmental exposures. These factors might also have contributed to the 10-fold higher prevalence of DCM in *TTN*tv-negative family members (4.45%) when compared to the population prevalence of DCM (.036%–.04%).[10] Clinical risk factors for DCM were present in some of these *TTN*tv-negative cases (i.e. phenocopies). Rare cardiomyopathy gene variants were found in five of the *TTN*tv-negative individuals, and it seems likely that additional unidentified rare variants could be present that segregate independently from the *TTN*tv in families.

There is emerging evidence that polygenic risk scores for DCM (DCM-PRS), derived from combinations of common genetic variants, are relevant even in suspected monogenic diseases. In a recent familial DCM study, mean values for a DCM-PRS (comprised of 28 variants) were higher in probands and in affected relatives when compared to healthy controls and unaffected relatives, respectively.[49] Further, two genome-wide association studies in DCM case-control and biobank cohorts reported enrichment of high DCM-PRS (comprised of .5–1.1 million variants) in rare variant-positive as well as rare variant-negative DCM cases.[50,51] Additionally, phenome-wide association studies and Mendelian randomization identified links between DCM risk and clinical parameters, including body weight and hypertension.[50,51] DCM-PRS was not assessed in our study and its potential contribution to *TTN*tv-related DCM remains to be investigated.

Limitations of our study need to be noted. Our family-based findings may not be directly applicable to sporadic DCM cases or *TTN*tv identified in the general population. The observational nature of this study is a further limitation, and a rigorous prospective evaluation of the impact of clinical risk factors on DCM severity and outcomes is needed. The impact of ancestry on *TTN*tv manifestation also remains to be clarified. Most clinical reports of *TTN*tv, including this study, have been performed in cohorts of predominant European ancestry; *TTN*tv have shown variable association with DCM in individuals of African ancestry,[52,53] and there is a paucity of data for other ancestry groups.

In familial DCM, variable penetrance and expressivity have been considered to be a characteristic of the underlying gene/variant. Here we provide new evidence that the onset of *TTN*tv-related familial DCM is not determined solely by the *TTN*tv but is also closely related to the individual patient environment. We suggest that aggressive intervention to identify and treat, or avoid, disease-exacerbating clinical risk factors is crucial, especially in the young. Given the high lifetime risk of DCM, continued medical surveillance of *TTN*tv-positive family members is indicated. The frequency of follow-up should be tailored according to age, sex, and risk factor burden. Further, our data provide new hope that disease prevention may be possible and pave the way for clinical intervention trials.

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Supplementary data

Supplementary data are available at *European Heart Journal* online.

Declarations

Disclosure of Interest

A.S.A. has received consulting fees from Bristol Myers Squibb (BMS), Tenaya Therapeutics and Biomarin, and receives research support from Novo Nordisk. E.A.A. is a founder and advisory board member (Personalis, Deepcell, Svexa, Candela, Parameter Health, Saturnus Bio), advisory board member (SequenceBio, Foresite Labs, Pacific Biosciences, Versant Ventures) and non-executive director (AstraZeneca, Svexa). E.E.B. is a consultant for Grey Genetics. H.B. receives lecture fees from Merck Sharp and Dohme, BMS, Amgen, and Pfizer and has received research funding from the Novo Nordisk Foundation. C.M. is a Novo Nordisk stock owner. M.G.C.L. is on the advisory board for Novo Nordisk and Takeda Pharmaceuticals, receives consulting fees from Medtronic, and payments from AstraZeneca, Boehringer Ingelheim, Bayer, Medtronic and Vifor. C.S.H. receives consulting fees and honoraria from Abbott Laboratories, is a board member of the International Society for Mechanical Circulatory Support, and is associated editor of the Journal of Heart Lung Transplantation. S.H. receives personal fees for independent scientific advice on early development in the field of heart failure for AstraZeneca, Ribocure, and CSL Behring, and receives research support from AstraZeneca and CSL Behring for early preclinical development in heart failure. C.Y.H. is on the advisory board and receives consulting fees from BMS, Lexicon, Tenaya Therapeutics, BioMarin Pharmaceutical Inc, Cytokinetics, Sanofi, Rocket, and Viz.ai. D.Z. is a member of the National Advisory Group for a study looking into Aboriginal women reducing the risk of diabetes and heart complications in pregnancy and is a Board member, Treasurer and Executive committee member of the Cardiac Society of Australia and New Zealand, as well as Chair of its Quality Standards Committee. P.S.M. receives support from Novartis, consulting fees from Boehringer Ingleheim, Novartis and AstraZeneca, payments from Boehringer Ingleheim, Abbott Laboratories, the Indian Society for Heart and Lung Transplantation, and the Congress of the Asian Society of Transplantation, along with being a shareholder in Infensa Bioscience and receiving perfusion modules from Transmedics. M.M. participates in the advisory board for Pfizer, Novartis, Novo Nordisk, Vifor, and AstraZeneca and holds an unrestricted research grant until 2023 from Pfizer. L. Monserrat is a shareholder in Health in Code. J.P.T. receives consulting fees from BioMarin Pharmaceutical Inc. K.E.W.S. participates in the advisory board of CSL and receives speaking honoraria from Pfizer and CSL. D.Z. is a member of the National Advisory Group for a study looking into Aboriginal women reducing the risk of diabetes and heart complications in pregnancy and is a Board member, Treasurer and Executive committee member of the Cardiac Society of Australia and New Zealand, as well as Chair of its Quality Standards Committee. C.A. participates in the steering committee for Novo Nordisk, Astra Zeneca, and Amgen. C.A.J. is involved with the National Society of Genetic Counselors Board of Directors and supported by Arvada Therapeutics, Lexeo Therapeutics, StrideBio Therapeutics, Eicosis Inc, and Tenaya Therapeutics. R.B.V. is on the advisory board and receives personal fees from Alnylam Pharmaceuticals, BMS, Chiesi, Cytokinetics, Pfizer, Sanofi, and receives funding from Health in Code, BMS, and Sanofi. B. Meder is involved with DGK eCardiology and receives various fees for consulting (Novo Nordisk, BMS, Cytokinetics), speaking (BMS, Novartis, Pfizer, AstraZeneca, DGK, Bayer, Amgen, BNK), testimony (BMS, Novartis, Pfizer, AstraZeneca, DGK, Bayer, Amgen, BNK), patents (Epigenetic biomarkers PCM heart failure) and has received equipment from Apple. J.R.G. participates in modest consulting for Avidity Biosciences, has an equity-sharing agreement with Prolaio Inc, and receives funding for clinical trials from Tenaya Therapeutics. V.N.P. receives fees from Lexeo Therapeutics, BioMarin Pharmaceutical Inc, Constantiam Biosciences, and Nuevocor. J.S.W. is involved with Cardiomyopathy UK and receives consulting fees from BMS, Pfizer, Foresite Labs, Health Lumen

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Data Availability

Data used in the cohort analyses are available upon reasonable request to the corresponding author and in accordance with consent and privacy requirements of referring centres.

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Ethical Approval

Study protocols were approved by the St Vincent's Hospital and relevant institutional Human Research Ethics Committees.

Pre-registered Clinical Trial Number

None supplied.

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