Title: Mortality Risk associated with Truncating Founder Mutations in Titin

Short title: Mortality associated with truncating TTN mutations

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ABSTRACT

Background: Truncating Titin variants (TTNtv) are the most prevalent genetic cause of dilated cardiomyopathy (DCM), found in up to 25% of familial cases. Moreover, TTNtv associated with DCM are estimated to be present in 0.5% of the general population. The prognosis of asymptomatic carriers of TTNtv is poorly understood, as TTNtv are associated with a highly variable phenotype. We aim to assess the natural history and clinical relevance of TTNtv by analysing standardised mortality ratios (SMR) in multigenerational pedigrees and in close relatives of present-day patients.

Methods and results: Haplotype and genealogical analyses of three recurrent TTNtv established them as founder mutations with pedigrees traced back to 18th century ancestors. Subsequently, the Family Tree Mortality Ratio method was used to compare all-cause mortality of subjects at an *a priori* 50% risk of carrying TTNtv to the general Dutch population. SMR were stratified for sex, age and calendar-period. Subgroups were compared with Poisson regression. In 20,522 person-years, overall mortality was not significantly increased (SMR 1.06, 95% CI 0.95-1.18, p=0.162). However, mortality was significantly increased in subjects living after 1965 (SMR 1.27, 95% CI 1.04-1.53, p=0.009) and aged ≥60 years (SMR 1.17, 95% CI 1.01-1.35, p=0.02). Similarly, SMR were calculated in parents of 128 present-day DCM probands with TTNtv using the reverse parent-offspring method. This showed overall excess mortality (SMR 1.26, 95% CI 1.07-1.48, p=0.003), driven by subjects aged ≥60 years.

Conclusions: The natural history of the analysed TTNtv shows a relatively mild disease course with significant excess mortality in elderly patients. With increasing life expectancy, TTNtv-associated morbidity and mortality will likely become more prevalent.

Key words: dilated cardiomyopathy, titin, natural history

INTRODUCTION

Dilated cardiomyopathy (DCM) is characterised by dilatation of the left ventricle and systolic dysfunction that is not proportional to coronary artery disease, hypertension or valve disease.[1] It is a major cause of end stage heart failure and the leading indication for cardiac transplantation.[2] Estimations of the prevalence of DCM range from 1:250 to 1:400.[3] The aetiology of DCM is diverse, including genetic, toxic, infectious, metabolic and endocrine causes, as well as inflammatory, infiltrative and autoimmune disease, pregnancy and tachyarrhythmia.[1, 2, 4] A potential genetic cause is identified in 30-40% of idiopathic DCM cases.[3, 5] Currently, more than 100 genes have been implicated in DCM, including *TTN*.[3, 5-7] *TTN* encodes the largest human protein, titin, an abundant structural, sensory, and signalling filament in muscle, consisting of Z-disc, I-band, A-band and M-band domains.[8] *TTN* truncating variants (TTNtv) constitute the most prevalent genetic cause of DCM, found in 19-25% of familial and 11-18% of sporadic cases.[5, 7, 9] TTNtv are also present in the general population, initially estimated at 1-2%.[7, 8, 10-12] However, this high prevalence sparked controversy regarding the pathogenicity of TTNtv as it exceeded the prevalence of DCM, complicating the interpretation of genetic testing and counselling of test results.[12] Subsequent estimations of the prevalence of DCM-associated TTNtv, based on position in the A-band and/or constitutional expression in the heart, were lower: 0.36-0.5%.[10, 13] This still means that up to 37.5 million people globally may carry a DCM-associated TTNtv and are potentially at an increased risk of developing heart failure[7] and cardiac arrhythmia.[14, 15] Recent studies found preclinical eccentric remodelling in asymptomatic TTNtv carriers [13] and observed a relatively good response to medical treatment of TTNtv-related DCM.[16, 17] These findings implicate that TTNtv carriers may benefit from early detection of cardiac involvement and treatment.

However, the prognosis of asymptomatic TTNtv carriers remains largely unknown due to reduced penetrance and highly variable disease severity. In this study, we sought to elucidate the natural history and clinical relevance of TTNtv by using the Family Tree Mortality Ratio (FTMR) method.[18, 19] Multigenerational pedigrees were constructed for three Dutch founder TTNtv located in the A-band and standardised mortality ratios (SMR) were determined, stratified by sex, age group and calendar period. Furthermore, as a representation of TTNtv carriers seen in current clinical practice, we analysed SMR in the parents of present-day DCM patients carrying TTNtv, stratified by sex and age group.

METHODS

Subject inclusion and genetic testing

This study was conducted in accordance with the principles laid out in the Declaration of Helsinki and in line with guidelines provided by ethics committees of the participating study centres. In this retrospective, multicentre study, we identified recurrent TTNtv among patients referred to the University Medical Centre Utrecht, Amsterdam University Medical Center, University Medical Centre Groningen, Erasmus University Medical Centre, Leiden University Medical Centre and Academic Hospital Maastricht, The Netherlands. Patients had been referred between 2012 and 2016 for genetic testing related to a diagnosis of DCM or family screening. Genetic testing was performed using next-generation sequencing or Sanger sequence analysis for the specific familial TTNtv. Extended haplotype analysis using intragenic SNPs was performed in probands and informative family members to assess whether the TTNtv were present on a shared genetic background, which would indicate a common ancestral origin.

Genealogical studies and FTMR analysis

We collected genealogical data using the official records of births, marriages and deaths. These data are well preserved in The Netherlands and have been reliably collected regardless of socioeconomic status from 1811 onwards.[19-21] The endpoints of the analyses were death or censoring in August 2012, when *TTN*-screening became available in The Netherlands. Complete multi-generational pedigrees were created by tracing ancestors of the probands throughout as many generations as possible or until all pedigrees of probands with the same mutation had been connected. This establishes transmission lines along which the TTNtv were passed on to next generations. All first-degree relatives of the individuals along the transmission lines were added to the pedigrees to create complete sibships. Based on a Mendelian model of inheritance, all individuals in these pedigrees are at an *a priori* 50% risk of having the TTNtv. All present day probands were excluded to avoid selection bias. Data on years of births and deaths were collected and person-years were calculated. As individuals on the transmission line were selected based on having lived long enough to pass on the TTNtv to the next in line, the years lived prior to passing on the TTNtv were not considered "at risk" of mortality. Therefore, the parental years lived before the birth of the next person on the transmission line were omitted from analysis to avoid "reproduction" bias. Using the Person-years program, observed all-cause mortality was compared to expected all-cause mortality, derived from public data available for the complete Dutch general population.[22] Analyses were stratified for sex, age and calendar period. The first year of life of all subjects was omitted in the analysis stratified for age periods as a sensitivity analysis, as registration of neonatal mortality in the 19th century may have been incomplete. The primary outcome was Standardised Mortality Ratio (SMR). [19-21]

Reverse parent-offspring analysis

To gain insight in the effects of TTNtv in the close relatives of probands with a wider range of TTNtv and to act as a validation cohort, we assessed the mortality of parents of present-day probands. From two larger centres (University Medical Centre Utrecht and Amsterdam University Medical Center), we identified DCM probands with TTNtv that had been classified as pathogenic or likely pathogenic.[23] Dates of births and deaths of both parents (*a priori* 50% risk carriers) were retrieved from official records. Parental years lived before birth of the probands were omitted to avoid reproduction bias. Moreover, to avoid double analysis, all subjects included in the reverse parent-offspring analysis were excluded from the analysis of the multigenerational pedigrees.

Statistical analysis

Statistical analysis was performed using SPSS Statistics version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). For normally distributed continuous variables, means were calculated and analysed using independent samples T-test. Medians and Mann-Whitney U test were used for non-normally distributed continuous variables. SMR (observed-to-expected mortality) was calculated, stratified for sex, age and calendar period, using the Person-years program.[22] The expected mortality was calculated by taking the total person-years at risk lived by the study population per sex, and age category and calendar period, and multiplying this by the corresponding mortality rate in the Dutch population, as previously described.[19-21, 24] The 95% confidence interval (CI) of the SMR was calculated assuming a Poisson distribution of the observed number of deaths and using exact limits.[18] Rate Ratios (RR) were calculated with Poisson regression.

RESULTS

Subject inclusion

A total of 24 DCM probands carrying the three recurrent TTNtv in the A-band were identified. Clinical characteristics of these probands are described in Table 1, details on genetic analysis are provided in Supplementary Table 1. The localization of the TTNtv in the gene is illustrated in Figure 1. Extended haplotype analysis showed each of these three TTNtv to be on a mutation-specific chromosomal background, indicating a founder effect for all three mutations (Supplementary Figure 1). A total of 128 DCM probands carrying 92 different TTNtv were identified for the reverse parent-offspring analysis (Figure 1 and Supplementary Table 2).

Family tree mortality ratio analysis

Results of pedigree analysis are summarised in Table 2 and pedigrees are depicted in Figure 2. Pedigree analysis successfully traced 18 (out of 24) probands to four pedigrees. Pedigrees A and B (p.(Glu25818*)) were considered to represent one family since haplotype analysis showed all probands to originate from a common ancestor. In total, 61 individuals on the transmission line and their 360 first-degree relatives were included in the multigenerational pedigrees, accumulating to a total of 20,522 person-years, during which 317 deaths were observed, of which 279 occurred after the age of one. There was no statistically significant overall excess mortality (SMR 1.06, 95% CI 0.95-1.18, p=0.162).

The SMR's according to sex, age categories and calendar periods are depicted in Figure 3 (further detail in Supplementary Table 3). The overall all-cause mortality risk was not different between males and females (RR 1.04, 95% CI 0.83-1.30, p=0.734).

However, stratification by age categories revealed that among the highest age group (≥60 years) overall excess mortality was statistically significant (SMR 1.17, 95% CI 1.01-1.35, p=0.020). The point-estimates for males and females aged ≥60 years were similarly directed, but did not reach significance (SMR: 1.13, 95% CI 0.92-1.37, p=0.118 and 1.21, 95% CI 0.97-1.49, p=0.080, respectively).

Stratification by calendar periods showed significantly increased mortality in subjects living between 1965 and August 2012 (SMR 1.27, 95% CI 1.04-1.53, p=0.009). Especially males had a strongly increased mortality risk after 1965 (SMR 1.43, 95% CI 1.09-1.83, p=0.006).

Stratification by mutation did not yield evident differences. Poisson regression comparing pedigrees A+B and C to D did not show differences in mortality rates (RR 1.11, 95% CI 0.8387-1.459, p=0.47).

Reverse parent-offspring analysis

Results of the reverse parent-offspring analysis are shown in Figure 4 (further detail in Supplementary Table 2). In 9,598 person-years, 147 deaths were observed corresponding with a significant overall excess mortality (SMR 1.35, 95% CI 1.14-1.59, p=0.0003). Stratification by age categories showed that excess mortality only reached significance among subjects aged ≥60 years (SMR 1.47, 95% CI 1.23-1.75, p=0.00001). Analyses according to sex showed that excess mortality occurred in both females and males aged ≥60 years (SMR 1.45, 95% CI 1.08-1.89, p=0.009 and SMR 1.29, 95% CI 1.00-1.56, p=0.046, respectively).

DISCUSSION

In this study, we investigated the natural history of TTNtv by analysing all-cause mortality. In 20,522 person-years in three multigenerational pedigrees, we observed that the overall SMR was not significantly increased. However, clear excess mortality occurred after 1965, predominantly in persons aged ≥60 years. The parents of present-day patients with TTNtv showed significant overall excess mortality, driven by persons aged ≥60 years. At least 35% of the reported causes of deaths in our analyses were likely related to TTN-associated DCM. Our results support a relatively mild disease course with excess mortality in the elderly.

The excess mortality in the elderly found in this study illustrates the clinical relevance of DCM-associated TTNtv and offers insight in the prognosis of TTNtv carriers. Previously, cohort studies have implicated TTNtv in both a relatively mild DCM-phenotype with good response to medical treatment,[16, 17] as well as a severe phenotype with high rates of heart transplantation, left ventricular assist device implantation, malignant arrhythmia and death.[7, 8] The FTMR method allowed us to investigate TTNtv-related mortality free from ascertainment bias, which is inherent to cohort studies and was recently observed in multiple cohorts of patients with inherited cardiac disease.[25] The SMR calculated in this study was calculated using complete sibships based on an *a priori* chance of carrying the TTNtv of 50%. Therefore, it should show 50% of the excess mortality. The natural history of TTNtv showed modest excess mortality compared with other FTMR studies (Supplementary Table 4). To illustrate, point estimates in age categories with significant excess mortality were relatively low compared to a previous FTMR study involving mutations in the myosin-binding protein C-3 gene, associated with hypertrophic cardiomyopathy.[20] Excess mortality in studies in two other genes associated with DCM (lamin A/C and phospholamban) also showed higher point-estimates, although it should be noted that

the former only analysed present day patients and did not investigate natural history.[26, 27] This supports a relatively mild disease course associated with TTNtv, in line with a recent report.[16] Prior to 1965, no significant overall mortality was observed, while after this time period there was a significant SMR of 1.27. This was confirmed by the overall excess mortality seen in our parent-offspring analysis, which acted as a validation cohort and gives additional insight in the effects of TTNtv close relatives of present-day patients. This may seem counterintuitive, as treatment of heart failure in the form of medical treatment and implantation of cardioverter defibrillators has rapidly emerged since the 1970's. However, the excess mortality becoming evident after 1965 may result from a loss of competing risks. This is supported by the increase in average life expectancy in the Dutch general population.[28] The increase in life expectancy among the elderly may have been subdued by the effect of the TTNtv. As shown in Supplementary Table 5, the observed mean age at the time of death for individuals aged 60 in the general population has steadily risen from 73.41 years for men and 74.06 years for women in 1861-1866, to 77.68 years and 82.82 years in 1961-1966.[28] Conversely, in the multigenerational pedigrees this varied over time, with an age of 70.6 years for men and 77.57 years for women in 1961-1966. A similar effect was previously reported in an FTMR study in the cyclin-dependent kinase inhibitor 2A gene, associated with melanoma.[24] Moreover, non-adherence to guidelines, comorbidities and polypharmacy have been shown to be important limitations in the optimal treatment of heart failure in elderly.[29, 30] With life expectancy continuing to increase, TTNtv-associated morbidity and mortality will likely become more prevalent. Furthermore, in-depth phenotyping of the probands in our study revealed high degrees of response to medical treatment similar to those reported previously by Jansweijer *et al.* (data not shown).[16] Therefore, dedicated

genetic screening and cardiologic follow-up of genotype positive, phenotype negative individuals is recommended.

Although our data support a modest disease course for TTNtv, our cohort and previous publications show that severe phenotypes do occur, even at young ages. Both genetic background and environmental factors including pregnancy, excessive alcohol consumption and chemotherapy,[31-36] have previously been suggested to explain this heterogeneity. In our study, one proband was found to carry a second pathogenic mutation and possible environmental factors were identified in a high proportion of patients. Future studies should be directed at further unravelling the disease mechanisms involved in expression and severity of TTNtv-associated DCM.

Study limitations

The SMR analyses were performed using an undisputable outcome, *i.e.* all-cause mortality. However, this inherently limits our analysis by omitting other aspects of disease burden. To ascertain the burden of TTNtv, causes of death were retrieved from family histories and pedigrees collected by clinical geneticists and cardiologists, shown in Supplementary Table 6. A possibly DCM-related cause of death was reported in 35% of patients.

As already noted, heart failure treatment, including implantable cardioverter defibrillators, has emerged since the 1970's. Therefore, the results of the SMR after this time-period may even be an underestimation of the true natural history. Concerning the FTMR analysis, as perinatal and early childhood mortality are most likely subject to under-registration, results for this age period may be subject to imprecision. Furthermore, thirteen parents of probands who were included in the reverse parent-offspring analysis, were excluded from the FTMR study to avoid double

analysis. As these were obligate carriers, deceased after 1965, the effect in this calendar period may be underestimated.

The reverse parent-offspring analysis is limited by the inclusion of only the parents of probands, excluding subjects who might not have reached reproductive age. Secondly, a wide variety of TTNtv were included, of which pathogenicity has not been formally proven. A small number of the included TTNtv are located outside of the A-band but, with the exception of two, all are in constitutively expressed exons. Finally, there is a small chance that some of the TTNtv used in this analysis occurred *de novo*, resulting in neither parent carrying the mutation. However, in practice this occurs only very rarely. These limitations may have led to an underestimation of the true effects in close relatives of present-day patients.

CONCLUSIONS

In the Dutch population, we identified three founder TTNtv located in the A-band region. We studied the natural history of founder TTNtv across age categories and calendar periods by performing FTMR analysis. This revealed significant excess mortality in subjects aged ≥60 years and subjects living after 1965. Additionally, we studied mortality in a reverse parent-offspring analysis, observing significant overall excess mortality due to deaths occurring among subjects aged ≥60 years. Our results indicate a relatively mild disease course with significant excess mortality only seen in elderly patients. However, with life expectancy continuing to increase, TTNtv-associated morbidity and mortality will likely become more prevalent. Based on our results and previous reports that indicate that TTNtv-associated DCM has a relatively good response to medical treatment, dedicated genetic screening and cardiologic follow-up of genotype positive, phenotype negative individuals is highly recommended.

Conflict of interest:

No relationships relevant to this study to disclose.

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TABLES

Table 1. Clinical characteristics in probands with founder TTNtv

		Probands founder TTNtv (N=24)	
Sex	Male	16/24	68%
Fulfilling DCM diagnostic criteria		22/24	92%
Median age at DCM diagnosis (25%-75% percentile) (years)		45 (36-52)	
Median age last follow-up (25%-75% percentile) (years)		54 (42-62)	
Additional variant in known DCM gene*		1/24	4%
	Likely pathogenic	0/24	0%
	Pathogenic	1/24	4%
Factor(s) possibly contributing to penetrance		9/24	38%
	Pregnancy	2/8	25%
	Alcohol abuse	1/24	4%
	Drug abuse	0/24	0%
	Chemotherapy	1/24	4%
	Hypo- or hyperthyroidism	1/24	4%
	Tachyarrhythmia	3/24	13%
	Mitral insufficiency	0/24	0%
	Coronary artery disease	1/24	4%
	(Suspected) myocarditis	1/24	4%
Rhythm disorders	Atrial fibrillation	5/24	21%
	Non-sustained ventricular tachycardia	11/24	46%
	Sustained ventricular tachycardia	4/24	17%

	Ventricular fibrillation	1/24	4%
Conduction disease	Atrioventricular block grade 1	2/24	8%
	Atrioventricular block grade 3	1//24	4%
	(incomplete) Left bundle branch block	2/24	8%
	(incomplete) Right bundle branch block	2/24	8%
Outcome	Stroke	2/24	8%
	Left ventricular assist device	3/24	13%
	Heart transplant	1/24	4%
	Aborted cardiac arrest or appropriate ICD-therapy	2/24	8%
	All-cause mortality	2/24	8%
	Heart-failure related death	1/24	4%
	Sudden cardiac death	0/24	0%
	Composite outcome	7/24	29%
Median age composite outcome (25%-75% percentile) (years)		48 (44-54)	

The clinical characteristics of the 24 probands carrying one of the three different founder TTNtv. The composite outcome consisted of implantation of a left ventricular assist device, heart transplantation, aborted cardiac arrest or appropriate ICD therapy, sudden cardiac death and heart failure related death.

*one proband carried an additional pathogenic mutation in *MYBPC3* (c.442G>A p.[Gly148Arg, Gly148fs]) , inherited from the parent not carrying the TTNtv.

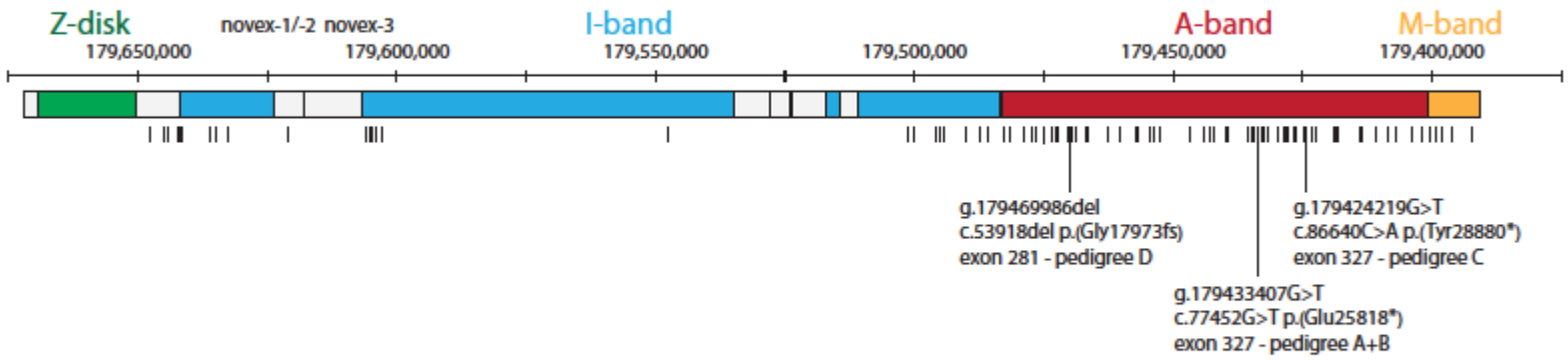
DCM, dilated cardiomyopathy; ICD, implantable cardioverter defibrillator; TTNtv, truncating variants in the titin gene.

Table 2. Founder TTNtv and results pedigree analysis

Mutation position in reference sequence NM_001267550.2	Probands identified	Pedigree (probands in pedigree)	Year of origin	Included subjects	Person-years	Deaths (deaths at age ≥1 year)	Median age of death (years, interquartile range)
c.53918del p.(Gly17973fs)	9	D (3)	1739	108	5,674	70 (67)	65 (37-78)
c.77452G>T p.(Glu25818*)	11	A (9) + B (2)	1776	231	11,046	176 (149)	61 (30-76)
c.86640C>A p.(Tyr28880*)	4	C (4)	1757	82	3,803	71 (63)	63 (36-73)
Total	24	18		421	20,522	317 (279)	62 (33-76)

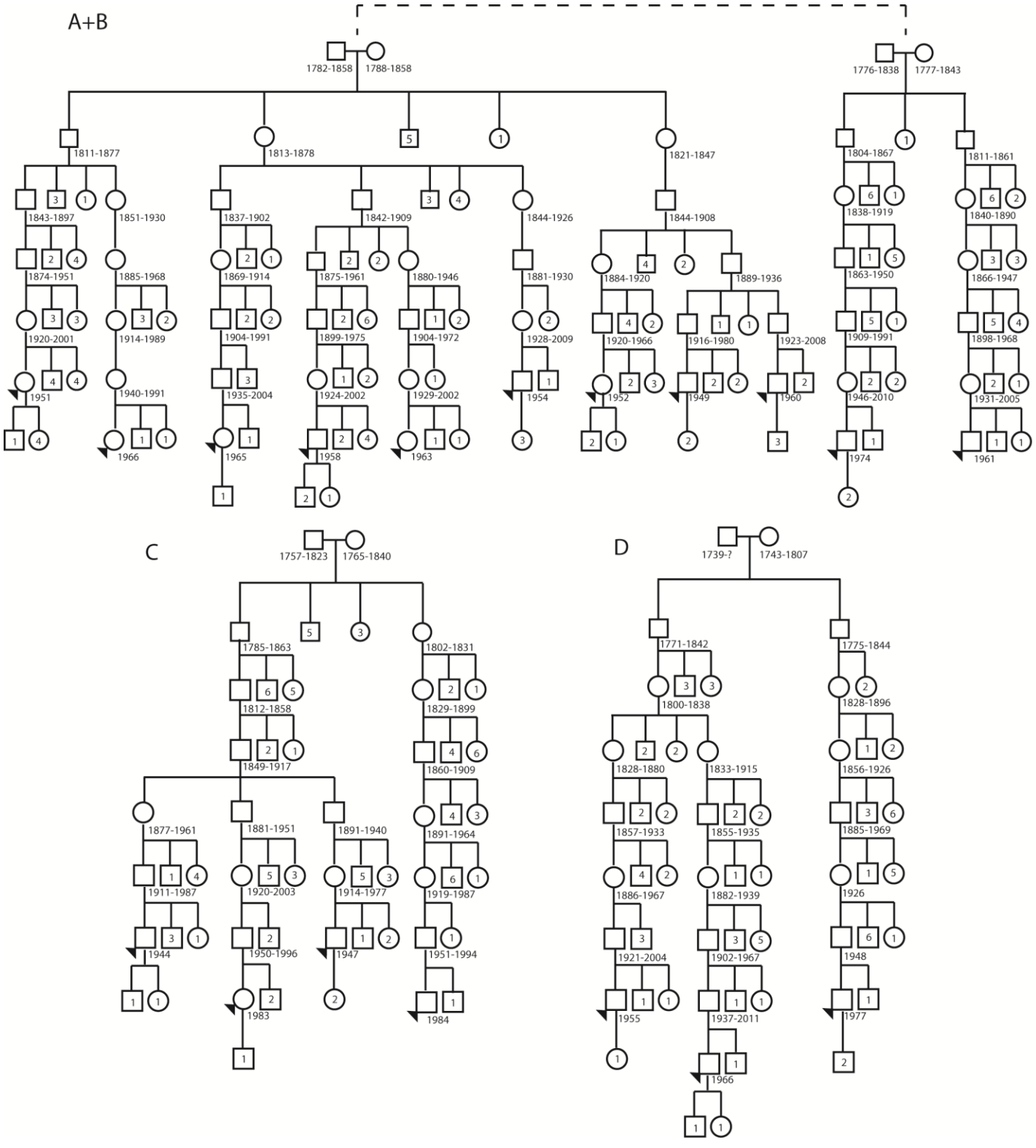
The three founder TTNtv included in our study with a summary of the results of genealogical analysis. The year of birth of the oldest subject in the pedigree is given as year of origin.

Figure 1. Schematic representation TTNtv within *TTN*



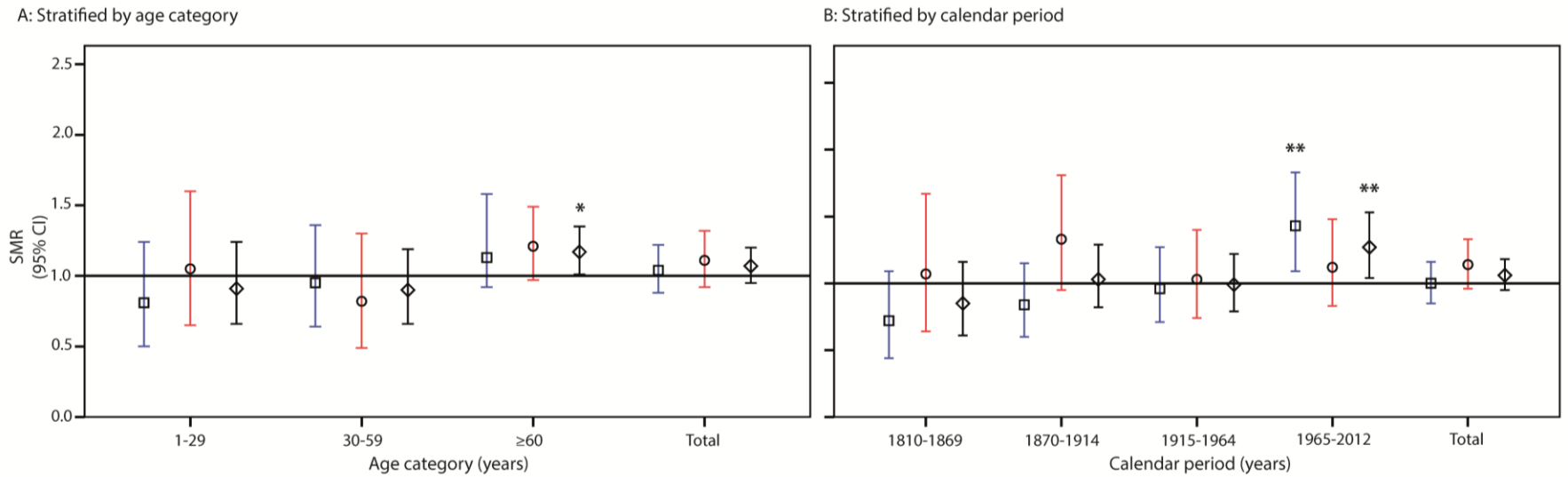
Schematic representation of the *TTN* gene, based on the Hg19 genomic position data included in the overview of *TTN* transcript and exon usage data presented by Roberts *et al.* (2015).[8] Included in the figure are the positions of the TTNtv included in the reverse parent-offspring analysis (indicated by small black bars) and the TTNtv founder mutations (large black bars with annotation of the exact mutation, exon number and pedigree).

Figure 2. Pedigrees



The three pedigrees included in our Family Tree Mortality Ratio analysis: A+B (c.77452G>T; p.(Glu25818*)), C (c.86640C>A; p.(Tyr28880*)) and D (c.53918del; p.(Gly17973fs)). Males are represented by squares, females by circles and probands have been indicated with an arrow. Year of birth and death are provided for individuals on the transmission lines; siblings are shown as numbers in the symbols corresponding to their sex.

Figure 3. Standardised Mortality Ratio analyses of multigenerational pedigrees

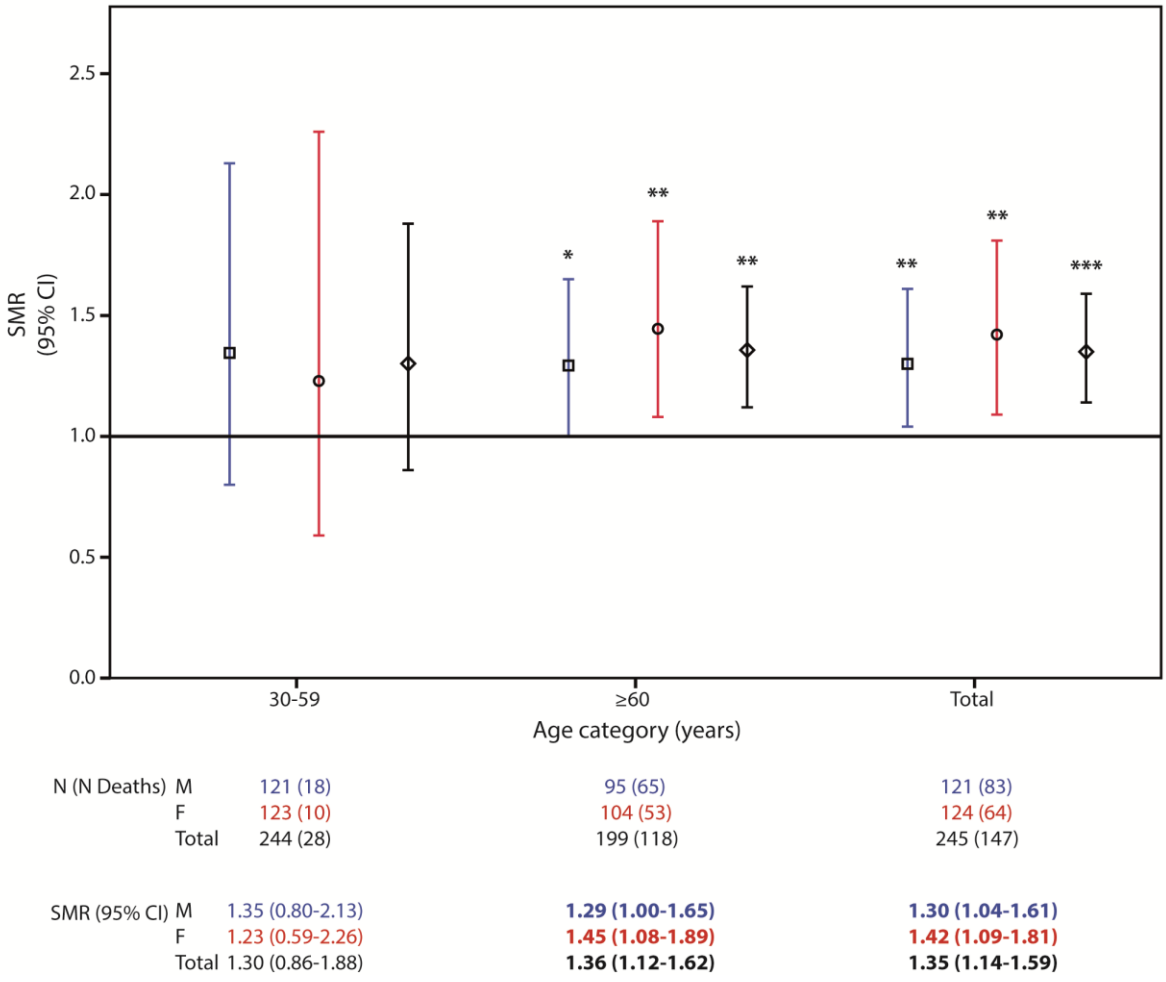


		1-29	30-59	≥60	Total	1810-1869	1870-1914	1915-1964	1965-2012	Total
N (N Deaths)	M	182 (21)	171 (30)	119 (101)	201 (152)	57 (21)	104 (39)	138 (49)	110 (60)	219 (169)
	F	170 (21)	152 (18)	109 (88)	181 (127)	52 (19)	103 (40)	120 (40)	103 (49)	202 (148)
	Total	352 (42)	323 (48)	228 (189)	382 (279)	109 (40)	207 (79)	258 (89)	213 (109)	421 (317)
SMR (95% CI)	M	0.81 (0.50-1.24)	0.95 (0.64-1.36)	1.13 (0.92-1.58)	1.04 (0.88-1.22)	0.72 (0.44-1.09)	0.84 (0.6-1.15)	0.96 (0.71-1.27)	**1.43 (1.09-1.83)**	1.00 (0.85-1.16)
	F	1.05 (0.65-1.60)	0.82 (0.49-1.30)	1.21 (0.97-1.49)	1.11 (0.92-1.32)	1.07 (0.64-1.67)	1.33 (0.95-1.81)	1.03 (0.74-1.40)	1.12 (0.83-1.48)	1.14 (0.96-1.33)
	Total	0.91 (0.66-1.24)	0.90 (0.66-1.19)	**1.17 (1.01-1.35)**	1.07 (0.95-1.20)	0.85 (0.61-1.16)	1.03 (0.82-1.29)	0.99 (0.79-1.22)	**1.27 (1.04-1.53)**	1.06 (0.95-1.18)

Standardised Mortality Ratios for the subjects included in the multigenerational pedigrees, stratified by age category (A) and calendar period (B), and further stratified by sex. Males are shown as squares with blue bars, females as circles with red bars and totals of both sexes as diamonds with black bars. Provided are the number of subjects subjects contributing person-years to the analysed stratum, number of observed deaths and the standardised mortality ratios, stratified for male (M) and female sex (F) as well as the totals.

CI, confidence interval; SMR, standardised mortality ratio. * SMR (versus general population) $p<0.05$, ** $p<0.01$.

Figure 4. Standardised Mortality Ratio analyses of parents of present-day probands



Standardised mortality ratios for the parents of 128 present-day probands, stratified by age category and further stratified by sex. Males are shown as blue bars, females as red bars and totals of both sexes as black bars. Males are shown as squares with blue bars, females as circles with red bars and totals of both sexes as diamonds with black bars. Provided are the number of subjects contributing person-years to the analysed stratum, number of observed deaths and the standardised mortality ratios, stratified for male (M) and female sex (F) as well as the totals. The 1-29 years age category was omitted in this figure as only one death occurred in it.

CI, confidence interval; SMR, standardized mortality ratio. * SMR (versus general population) $p<0.05$, ** $p<0.01$, *** $p<0.001$.