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Pathogenic *RBM20*-variants are Associated With a Severe Disease Expression in Male Patients With Dilated Cardiomyopathy

Short title: Severe DCM in males carrying pathogenic *RBM20*-variants

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Abstract

Background

As pathogenic variants in the gene for *RBM20* appear with a frequency of 6 % among Danish patients with dilated cardiomyopathy (DCM), it was the aim to investigate the associated disease expression in affected families.

Methods and Results

Clinical investigations were routinely performed in DCM index-patients and their relatives. In addition, ≥ 76 recognized and likely DCM-genes were investigated. DNA-sequence-variants within *RBM20* were considered suitable for genetic testing when they fulfilled the criteria of (a) being pathogenic according to the ACMG-classification, (b) appeared with an allele frequency of $< 1:10.000$ and (c) segregated with DCM in ≥ 7 affected individuals.

A total of 80 individuals from 15 families carried five different pathogenic *RBM20*-variants considered suitable for genetic testing. The penetrance was 66% (53/80) and age-dependent. Males were both significantly younger and had lower ejection fraction at diagnosis than females (age: 29 ± 11 versus 48 ± 12 years; $p < 0.01$. Ejection fraction: 29 ± 13 versus $38 \pm 9\%$; $p < 0.01$). Furthermore, 11 of 31 affected males needed a cardiac transplant while none of 22 affected females required this treatment ($p < 0.001$). Thirty percent of *RBM20*-carriers with DCM died suddenly or experienced severe ventricular arrhythmias while no adverse events were identified among healthy *RBM20*-carriers with a normal cardiac investigation. The event-free survival of male *RBM20*-carriers was significantly shorter compared to female carriers ($p < 0.001$).

Conclusions

The disease expression associated with pathogenic *RBM20*-variants was severe especially in males. The findings of the current study suggested that close clinical follow-up of *RBM20*-carriers is important which may ensure early detection of disease development and thereby improve management.

Keywords

Dilated cardiomyopathy; Sudden cardiac death; Genotype-phenotype correlations; *RBM20*-variants.

Clinical Perspectives

What is new

- This study represents the most comprehensive investigation to date of the disease expression in 80 carriers of pathogenic DNA-sequence variants in the gene for *RBM20*
- Sixty percent of all male *RBM20*-carriers experienced a major cardiovascular event before the age of 40 years while this happened in less than 5% of females
- Only male carriers developed end-stage heart failure requiring cardiac transplantation
- Pathogenic *RBM20*-variants were associated with a severe disease expression which appeared to be sex specific since males had a significantly shorter event-free survival rate than female carriers

Clinical Implications

- The severe disease expression in carriers of pathogenic *RBM20*-variants, suggested that close clinical follow-up of affected as well as unaffected individuals is warranted to ensure adequate and timely treatment
- Primary prophylactic ICD-treatment may be considered in carriers of pathogenic *RBM20*-variants once they develop DCM, since one third of the affected individuals either died suddenly or experienced episodes of ventricular fibrillation/sustained ventricular tachycardia

Introduction

Dilated cardiomyopathy (DCM) is a condition characterized by unexplained dilation and impaired systolic function of the left ventricle (LV).¹ The most common presentation is with symptoms of heart failure (HF), while ventricular arrhythmias and sudden cardiac death (SCD) are less frequent as the initial manifestation of the disease.¹ The prognosis has improved considerably over the past decades due to better medical treatment, use of cardiac resynchronization therapy and application of implantable cardiac defibrillators (ICD).² However, DCM remains to be one of the most frequent causes of end-stage HF and heart transplantation (HTx).³

The disorder has an estimated prevalence of 1:2500 and a familial appearance (>1 affected individual) in 30-50% of cases.⁴⁻⁶ In the context of familial disease DCM is most often transmitted by autosomal dominant inheritance and genetic investigations have reported pathogenic sequence variants in more than 100 different genes.⁷ So far, disease-associated DNA-sequence-variants have most frequently been identified in the giant gene for Titin (*TTN*) encoding part of the cytoskeleton, sarcomeric protein genes and the gene for lamin A/C which encodes a protein of the nuclear envelope.⁷

Recently, the gene for ribonucleic acid binding protein motif 20 (*RBM20*) was reported as the first DCM gene with regulatory properties that influences the posttranslational splicing of variety of genes including *TTN*.⁸

So far, only few studies of *RBM20* have reported about the relationship between genotype and phenotype.⁸⁻¹² The clinical information from these investigations has been limited by

small numbers of individual patients (index-patients) or families with few affected relatives and a short period of follow-up. It was the aim of the study to substantiate our knowledge about the disease expression associated with pathogenic DNA-sequence-variants in *RBM20* by clinical investigations of a significant number of affected individuals followed for a considerable period of time.

Methods

The data, analytic methods, and study materials will be made available on reasonable request to other researchers for purposes of reproducing the results or replicating the procedures.

Study Cohort

This investigation complied with the Declaration of Helsinki. It was approved by the local ethics committee (S-20140073) and the Danish data protection agency (14/17347). Informed consent was obtained from all participants.

Patients and relatives were included in the period from 2011 to 2017 at three tertiary referral University Hospitals of which one was also a transplant center. The catchment area of the hospitals included the geographical areas of Funen and Jutland which represented 54% (3.122.253/5.778.570 individuals) of the Danish population in 2017.¹³ The study cohort consisted of unrelated index-patients with Caucasian ethnicity and a diagnosis of DCM who were shown to carry pathogenic DNA-sequence-variants in the gene for *RBM20* following genetic investigations. Clinical data of the patients were collected retrospectively from their initial diagnosis of DCM until inclusion in the study and from then, prospectively until their most recent follow-up, their time of death or HTx.

In addition, their relatives at risk of disease development, who accepted the offer of predictive genetic testing and were shown to carry the pathogenic DNA-sequence-variant within *RBM20* of the index-patient, were also included in the study.

Information about family members who died before the current investigation was obtained retrospectively by reviewing available hospital notes and autopsy reports.

Information from each individual participating in the study was collected at multiple time points and included: (1) symptoms of cardiac disease, (2) results of ECG- and Holter-recordings, (3) results of echocardiography, (4) implantation of pacemaker or defibrillator (5) disease complications including ventricular arrhythmias, HTx, and (6) all-cause mortality.

Control Cohort

The control cohort consisted of patients with familial DCM defined as the presence of >1 affected individual following clinical investigations of relatives at risk of having inherited the condition. Furthermore, genetic investigations of ≥ 76 recognized and likely DCM-genes (see below) had been unable to identify any pathogenic or likely pathogenic DNA-sequence-variants. This cohort of patients fulfilled the same diagnostic criteria as the study cohort and was included at the same time and location as carriers of pathogenic *RBM20*-variants.

Echocardiography and Diagnostic Criteria

All individuals in the study- and control- cohort underwent echocardiography included standard two-dimensional measurements of left ventricular end-diastolic diameter (LVEDd) and left ventricular ejection fraction (LVEF) by Simpson's bi-plane method. Dimensions were corrected for age and body surface area (BSA) according to the formula of Henry [(LVEDd)(percent predicted) = measured LVEDd/predicted LVEDd X 100; predicted LVEDd = $[45.3 \times \text{BSA}^{0.3}] - [0.03 \times \text{age}] - 7.2$].¹⁴

DCM was diagnosed in accordance with generally accepted criteria when echocardiography identified unexplained left ventricle dilation and impaired contractile performance with a LVEDd >112% predicted for age and BSA and a LVEF $\leq 45\%$.¹

Coronary disease was excluded in index-patients >35 years of age at diagnosis by the use of coronary angiography or cardiac computed tomography.¹⁵ In addition, patients who were pregnant or had hypertension, a history of alcohol abuse, heart valve disease, congenital heart disease, autoimmune, endocrine, metabolic or neuromuscular disease known to be associated with HF were not included in the study.

RBM20-carriers who experienced SCD <50 years of age were also considered to have DCM.¹⁵

Sudden Cardiac Death (SCD)

SCD was defined in accordance with generally accepted criteria as a sudden and natural unexpected death. In un-witnessed cases SCD was defined as the cause of death in a person last seen alive and functioning normally 24 hours before dying and in witnessed cases as an acute change in cardiovascular status within one hour of the time to death.¹⁶

Ventricular Arrhythmia (VA)

VA was defined as documented episodes of (a) sustained ventricular tachycardia (VT) lasting >30 seconds requiring cardioversion, (b) ventricular fibrillation (VF) or (c) SCD.

Genetic Investigations

All index-patients in both the study and control cohort underwent genetic investigations by use of Illumina HiSeq NGS technology. Three different laboratories were used which all fulfilled the requirements for clinical diagnostic testing according to the UNE-EN ISO 15189 quality standard.¹⁷⁻¹⁹ The number of genes investigated varied from 76 to 242 and depended solely on the diagnostic gene panels provided by the individual laboratory (Table 1S).

Once a pathogenic DNA-sequence-variant was identified in the index-patient, relatives were offered predictive testing by use of Sanger Sequencing of the specific variant.

Filtering and Classification of DNA-Sequence-Variants

Sequence variants were filtered and classified according to the consensus recommendations from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) as pathogenic (P), likely pathogenic (LP), variant of unknown significance (VUS), benign or likely benign.²⁰ The initial raw filtering and classification was obtained by use of the software Ingenuity Variant Analysis (IVA, Qiagen®) and Intervar (Wang Genomics Lab®).²¹⁻²³ Subsequently, all P/LP variants as well as VUS were reassessed individually and reclassified according to ACMG criteria, following co-segregation analysis, literature- and database- review.²⁴⁻²⁸ *RBM20*-variants identified within the Arginine-Serine rich domain of exon 9 were considered to fulfil the hotspot ACMG-criteria (PM1) since previous investigations have reported identical pathogenic variants in the same domain and no benign variants have been reported so far. Evidence of segregation was considered as strong when appearing in ≥ 7 affected individuals, moderate when appearing in ≥ 5 and supporting when appearing in ≥ 3 .²⁹

Variants classified as pathogenic according to ACMG were considered to be disease associated and suitable for genetic testing when they:

- (a) Appeared in \geq seven individuals fulfilling DCM diagnostic criteria based on the findings in the current study and previous reports.^{24-27, 30}
- (b) Occurred with an allele frequency $< 1:10.000$ in the Genome Aggregation Database (gnomAD).^{28, 31}

Haplotype Analysis

To investigate if common founders of families carrying identical pathogenic *RBM20*-variants were present, haplotype analyses were made by the use of Sanger sequencing of three single nucleotide polymorphisms (SNP) in the three-prime-untranslated-region (3-UTR) of *RBM20* (genomic positions: 112596184/c.*448C>T; 112596570/c.*834G>T and 112598602/c.*2866C>T). All SNPs were localized within a distance of less than 27.000 nucleotides from the pathogenic *RBM20*-variant. On average three carriers of the pathogenic variant and one non-carrier from each family were selected for haplotype analysis. The primers used for haplotype analysis are available upon request.

Statistics

Continuous variables were reported as mean \pm standard deviation (SD) for normally distributed data or otherwise as median and interquartile range (IQR). Categorical variables were reported as numbers and percentages. Comparisons of groups were made by use of students T-test or the Mann-Whitney test for continuous variables where appropriate and χ^2 - or Fishers exact-test were used where appropriate for categorical variables. Comparisons of more than two groups with normally distributed data were made by the use of one-way ANOVA test and the Bonferroni test for pairwise comparison. Comparisons of more than two groups with non-parametric data were made by the use of Kruskal Wallis test and Dunn's test for pairwise comparison (Bonferroni). The follow-up period in survival analyses lasted from the date of birth until the first episode of VA, HTx, death from HF (composite endpoint) or the date of the most recent follow-up (censoring). Event rates were estimated using the Kaplan-Meier method and compared using the log-rank test. Furthermore, univariate cox

proportional hazard regression was used to calculate hazard ratios (HR) and 95% confidence intervals (CI). Family structure was taken into account by estimating robust clustered standard errors. P-values <0.05 were considered to be statistically significant. Statistical analyses were performed by the use of software from STATA (version 15).

Results

Genetic Investigations

Initially clinical and genetic investigations of ≥ 76 potential DCM related genes were performed in 111 DCM-index patients including 436 of their relatives at risk of having inherited the condition.³² Familial DCM occurred in 34% (38/111) of cases. A total of 14 different pathogenic DNA-sequence-variants suitable for genetic testing were identified in 19 apparently unrelated index-patients (17%) with a frequency of 45% (17/38) in familial DCM and 3% (2/73) in sporadic DCM. The frequency of pathogenic *RBM20*-variants in this cohort of patients was 6% (7/111) and appeared in familial DCM only. Following these results, genetic investigations were routinely offered to all consecutive index-patients with familial DCM and an additional eight *RBM20* families were identified and included in this study.

The genetic investigations identified five different recognized pathogenic *RBM20*-variants suitable for genetic testing in 15 families with a total of 80 carriers (Figure 1 and Table 1: p.Arg634Gln, p.Arg636His, p.Arg636Ser, p.Pro638Leu and p.Glu913Lys).^{6, 8-12}

Ten index-patients were shown to carry the same p.Arg636Ser amino acid substitution. Seven of these families were available for haplotype analyses which revealed co-segregation between three intra-genic SNPs of *RBM20* and p.Arg636Ser. These findings suggested a common disease allele and thereby a common founder of the pathogenic variant.

In family B the index-patient and one affected relative carried the recognized disease-associated pathogenic *RBM20*-variant p.Arg636Ser as well as a VUS within the DCM gene for *BAG3* (p.Arg71Trp) which appeared with a frequency of 1:7070 in gnomAD (Table 1).³³

An additional four index-patients were shown to carry four different VUS of *RBM20* (Table 1: p.Met196Val, p.Arg392Trp, p.Asp674Gly and p.Pro1039Ser). Three of these index-patients also carried a pathogenic truncating *TTN* variant or a VUS in recognized DCM genes for either *ACTC1* or *TNNI3* (Table 1 and Figure 1S).³⁴⁻³⁶ Unfortunately, the number of affected individuals in these families was too small to draw conclusions about the potential pathogenicity of these sequence variants and therefore they were excluded from the analyses in this study.

Clinical Investigations of *RBM20*-carriers

Age at Diagnosis and Symptoms

Clinical investigations of 80 individuals carrying pathogenic *RBM20*-variants within 15 families identified a total of 53 affected individuals including index-patients, which resulted in a penetrance of 66% (53/80). They were followed for a median period of 86 months (24 – 150 months) from their first clinical evaluation and were diagnosed with DCM at their initial investigation (n=49) or developed the condition during follow-up (n=4). The mean age at diagnosis of index-patients and affected relatives was 40 ± 15 and 35 ± 15 years respectively. Most affected individuals were diagnosed between 30 and 39 years of age (Figure 2 and Table 2). Twenty-seven individuals (34%) had a normal phenotype at their most recent follow-up at a mean age of 38 ± 17 years (males: 37 ± 17 years; females: 40 ± 17 years) (Figure 1, 3 and Table 2).

The total number of relatives carrying pathogenic *RBM20*-variants without symptoms of cardiac disease was 44 of which 17 (39%) were diagnosed with DCM following clinical family screening (Figure 3). These asymptomatic individuals with DCM were identified at a

significantly younger age compared to 16 symptomatic relatives who were diagnosed with DCM because of cardiac symptoms before family screening was initiated (29 ± 12 vs. 43 ± 16 years, $p=0.02$) (Table 2S).

An additional five relatives who were obligate carriers of pathogenic *RBM20*-variants died suddenly as the initial symptom of disease at an average age of 35 ± 6 (Figure 1: Family D: III.4 and III.6. Family F: II.2 and II.3. Family H: II.1). They all underwent autopsy and received a post-mortem diagnosis of DCM. According to the autopsy report of individual FII.3 her heart was enlarged with a dilated LV and a weight of 475 g (0.8% of her body weight). The histology was characterized by myocyte hypertrophy and fibrosis.

Unfortunately details of the autopsies in the remaining four SCDs were unavailable except for the main conclusion.

Echocardiography

Echocardiography of all carriers of pathogenic *RBM20*-variants with DCM at initial diagnosis revealed a mean LVEDd and LVEF of 65 ± 9 mm and 32 ± 12 %, respectively (Table 3).

Asymptomatic individuals who fulfilled DCM diagnostic criteria and identified by clinical investigations of relatives had a significantly better LVEF than relatives who had been referred due to symptoms (LVEF: 38 ± 8 vs. 27 ± 12 %, $p=0.03$) (Table 2S). There was no significant change in LVEF (32 ± 12 vs. 32 ± 13 %; $p=0.76$) or LVEDd (66 ± 10 vs. 68 ± 10 mm; $p=0.11$) of *RBM20*-carriers with DCM after an average period of seven years of follow-up.

Arrhythmias

Two individuals had supraventricular arrhythmias at diagnosis, while ten developed atrial fibrillation during follow-up. One individual developed a sick sinus node syndrome at the age of 51. Five individuals developed left bundle branch block (LBBB). No one was diagnosed with advanced atrioventricular conduction disease.

Three individuals were successfully resuscitated from a cardiac arrest due to VF while two individuals had episodes of sustained VT requiring cardioversion. Five of 16 patients treated with a primary prophylactic ICD received appropriate shock at a mean age of 40 ± 6 years and a median follow-up period of five years. Two of these individuals underwent HTx at a later stage. One individual who had been followed for eight years with a LVEF of 30%, died suddenly at the age of 33. An additional, five individuals also died from SCD as mentioned above. Most affected individuals experienced their first episode of VA between 30 and 39 years of age (Figure 2).

In total, 16 of the 53 individuals with DCM (30%) experienced at least one episode of severe VA. There was no significant difference in LVEF at diagnosis among patients with episodes of VAs compared with DCM patients without VAs (28 ± 13 vs. 34 ± 12 %; $p=0.19$), while the mean LVEF preceding an episode of sustained VT or VF was 30 ± 12 % (median 30%, range: 10 – 47%). However, 36% (4/11) of the patients with DCM who experienced a VA had a LVEF > 30%. There was no difference in HF therapy with ACE-inhibitors and beta-blockers between patients with and without VAs ($p=0.69$ and $p=0.13$, respectively).

RBM20-carriers with a normal echocardiography did not have symptoms or documented episodes of VAs by Holter-recordings.

Sex Specific Disease Expression

Eleven males from eight different families and no females underwent HTx due to end-stage HF ($p < 0.01$) at a mean age of 33 ± 16 years (Table 3). Four teenagers from three different families received a transplant at the age of 13 (II.2, family R), 14 (III.2, family G), 17 (III.5, family H) and 18 (III.7, family H) (Figure 1).

Males were diagnosed at a significantly younger age than females (29 ± 11 vs. 48 ± 12 years, $p < 0.001$). They also had a lower LVEF at diagnosis (29 ± 13 % vs. 38 ± 8 %, $p < 0.009$), while the difference in LVEDd at diagnosis was non-significant when indexed for BSA (Table 3).

Males were significantly younger when experiencing their first episode of VA (37 ± 5 vs. 54 ± 16 years, $p = 0.006$). By the age of 40 years 60% males had either received a cardiac transplant or experienced VA while this happened in less than 5% of females (Figure 4A).

However, there was no significant sex specific difference in the number of VA episodes (Table 3). Affected males also died or experienced a disease complication significantly earlier in life than affected females (log-rank; $p < 0.001$. HR 24.05; 95% CI, 2.98 – 196.21; $p = 0.003$) (Figure 4A). There were no significant differences in the number individuals who were identified by family screening, their NYHA class, pharmacological HF treatment, or the frequency of ICD implantations between sexes (Table 3). Adjusting for family structure did not change the significance levels in the statistical analyses (data not shown).

Disease Expression Among Affected Relatives

Affected relatives who were asymptomatic at diagnosis were diagnosed with DCM at a younger age, had a higher LVEF at diagnosis (38 ± 8 % vs. 27 ± 12 ; $p = 0.03$), and fewer episodes of VAs during follow-up (6 vs. 52%; $p = 0.008$) than affected relatives who were symptomatic at diagnosis (Table 2S). Two of the asymptomatic relatives at diagnosis

deteriorated rapidly during follow-up and underwent HTx at the age of 17 and 19 years respectively (Table 2S). There were no significant differences between index-patients and their affected relatives regarding pharmacological HF treatment, frequency of ICD implantations, VAs and HTx during follow-up (Table 2S).”

Comparison of the Disease Expression Among DCM Patients Carrying Pathogenic RBM20 and Patients Having Familial DCM of Unknown Genetic Etiology

The disease expression among carriers of pathogenic *RBM20*-variants and patients with familial disease in whom the genetic investigations failed to identify any pathogenic or likely pathogenic variants was compared.

Male patients (n=31) with DCM carrying pathogenic *RBM20*-variants were significantly younger at diagnosis (29 ± 11 vs. 49 ± 16 years, $p < 0.001$) than males (n=30) with familial DCM of unknown genetic etiology (Table 4). In addition, they had a significantly shorter event-free survival than males with familial DCM of unknown genetic etiology (log-rank; $p = 0.001$. HR 3.47; 95% CI, 1.58 – 7.60; $p = 0.002$) (Figure 4B). There were no further differences in the clinical characteristic, pharmacological HF treatment or frequency of ICD implantations between males in the two groups.

Female patients (n=22) with DCM carrying pathogenic *RBM20*-variants were significantly older at diagnosis than females (n=14) with familial DCM of unknown genetic etiology (48 ± 12 vs. 33 ± 16 years, $p = 0.003$). There were no further significant differences in clinical characteristics, pharmacological HF treatment, frequency of ICD implantations or event-free survival between females in the two groups (log-rank; $p = 0.19$. HR 0.46; 95% CI, 0.14 – 1.51; $p = 0.20$) (Supplementary material: Table 3S and Figure 2S).

Discussion

Genetic Investigations and Penetrance

Five recognized pathogenic *RBM20*-variants were identified of which the p.Arg636Ser amino acid substitution appeared in more than two thirds of all *RBM20*-carriers.^{6, 8-12} Haplotype analyses using three SNPs localized in close proximity to the pathogenic *RBM20*-variant suggested that this variant was likely to have originated from a common founder.

A number of rare sequence variants were identified in *RBM20* as well as in additional recognized DCM genes. Although they were absent in controls the number of affected carriers of these variants were too small to determine their possible impact on disease expression and therefore, they were considered unsuitable for genetic testing. Future studies may help to determine the impact of these variants and also clarify if DCM in the context of *RBM20*-disease may be explained by di-genic inheritance due to the appearance of variants in more than one recognized DCM gene as seen in four index patients of this study (Table 1 and Figure 1S).

The penetrance of DCM among carriers of pathogenic *RBM20*-variants was 66% including index-patients and depended on age and sex with males being diagnosed at a significantly younger age than females. Remarkably, seven young males and no females developed DCM in their teens necessitating HTx in four individuals before the age of 19.

Clinical Investigations and Disease Expression

The disease expression was severe since one third of affected carriers died suddenly or experienced at least one episode of VF/sustained VT during seven years of follow-up. In

addition, 21% received a cardiac transplant. Again, the disease expression was significantly worse among males in whom 60% experienced a major cardiac event before the age of 40 years while this occurred in less than 5% of females (Figure 4A).

It was evident that male DCM patients carrying pathogenic *RBM20*-variants had a significantly shorter event-free survival than males with familial DCM of unknown genetic etiology (Figure 4B). Furthermore, female *RBM20*-carriers were older at diagnosis than both females with familial DCM of unknown genetic etiology and male *RBM20*-carriers.

This sex specific disease expression may be explained by yet unidentified modifying genetic variants, which may protect female carriers from adverse events in addition to differences in lifestyle, hormonal status and overall genetic constitution. In this context, it is of interest that a similar sex specific disease expression has been reported to be associated with pathogenic variants in the gene for lamin A/C, in which males appeared to have more episodes of malignant VAs and a higher frequency of end-stage HF than females.³⁷

The fact that males had a lower LVEF at diagnosis than females may well explain the high frequency of HTx and SCD among male *RBM20*-carriers. Furthermore, the absence of VA and symptoms of disease in *RBM20*-carriers with a normal cardiac investigation also suggested that VA were related to impaired LV function. However, VAs also appeared in four individuals with a LVEF between 30-47%, suggesting that other factors than impaired LV function may be associated with VAs. Interestingly, the results of a recent investigation of a *RBM20* knock-out mouse model showed that loss of the protein for *RBM20* disturbed Ca²⁺ handling and lead to a more pro-arrhythmic Ca²⁺ release from the sarcoplasmic reticulum which may explain the high frequency of VAs.³⁸ In order to substantiate these

findings, additional experimental studies of genetically modified animal models expressing pathogenic missense variants identified in humans would be required to provide a basis for future pharmacological intervention studies.

Clinical implications

Based on the findings in this and previous studies pathogenic variants within *RBM20* appeared to be associated with an adverse prognosis in both index-patients and their relatives.

The finding of DCM in 39% of otherwise asymptomatic *RBM20*-carriers illustrated the importance of family screening. These individuals were identified 14 years earlier than relatives who had been diagnosed with DCM due to symptoms of HF. The diagnosis of asymptomatic individuals with DCM allowed initiation of pre-symptomatic anti-congestive medical therapy which may hopefully postpone the development of severe LV dysfunction, VA and the need for HTx.

Close clinical surveillance of *RBM20*-carriers from the age of 10 appeared important since several individuals developed end-stage HF in their teens while few carriers experienced adverse complications beyond the age of 70 years.

Limitations

The study was conducted at three tertiary referral University Hospitals of which one was also a transplant center which may have introduced referral bias towards more severely affected individuals.

Haplotype analyses suggested that the most frequent pathogenic *RBM20*-variant (p.Arg636Ser) in the cohort was likely to have arisen from a common ancestor. This may have represented a potential confounder towards a more uniform disease expression among

carriers due to a common genetic background. However, the disease expression associated with the p.Arg636Ser variant observed in this study appeared to be the same as reported previously which favored an effect of the pathogenic *RBM20*-variant by itself more than a potential effect of common ancestry.⁸

The fact that males with familial DCM of unknown genetic etiology were significantly older at diagnosis compared males with DCM carrying pathogenic *RBM20*-variants may have introduced a confounder because the development of their condition may have been influenced by a longer exposure to environmental and lifestyle risk for developing DCM.

Conclusion

Pathogenic *RBM20*-variants appeared to be associated with a severe disease expression and an early onset especially in males. These findings suggested that clinical and genetic investigations are important to identify patients at high risk of developing disease complications in order to initiate adequate and timely treatment.

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Conflict of Interest

None declared

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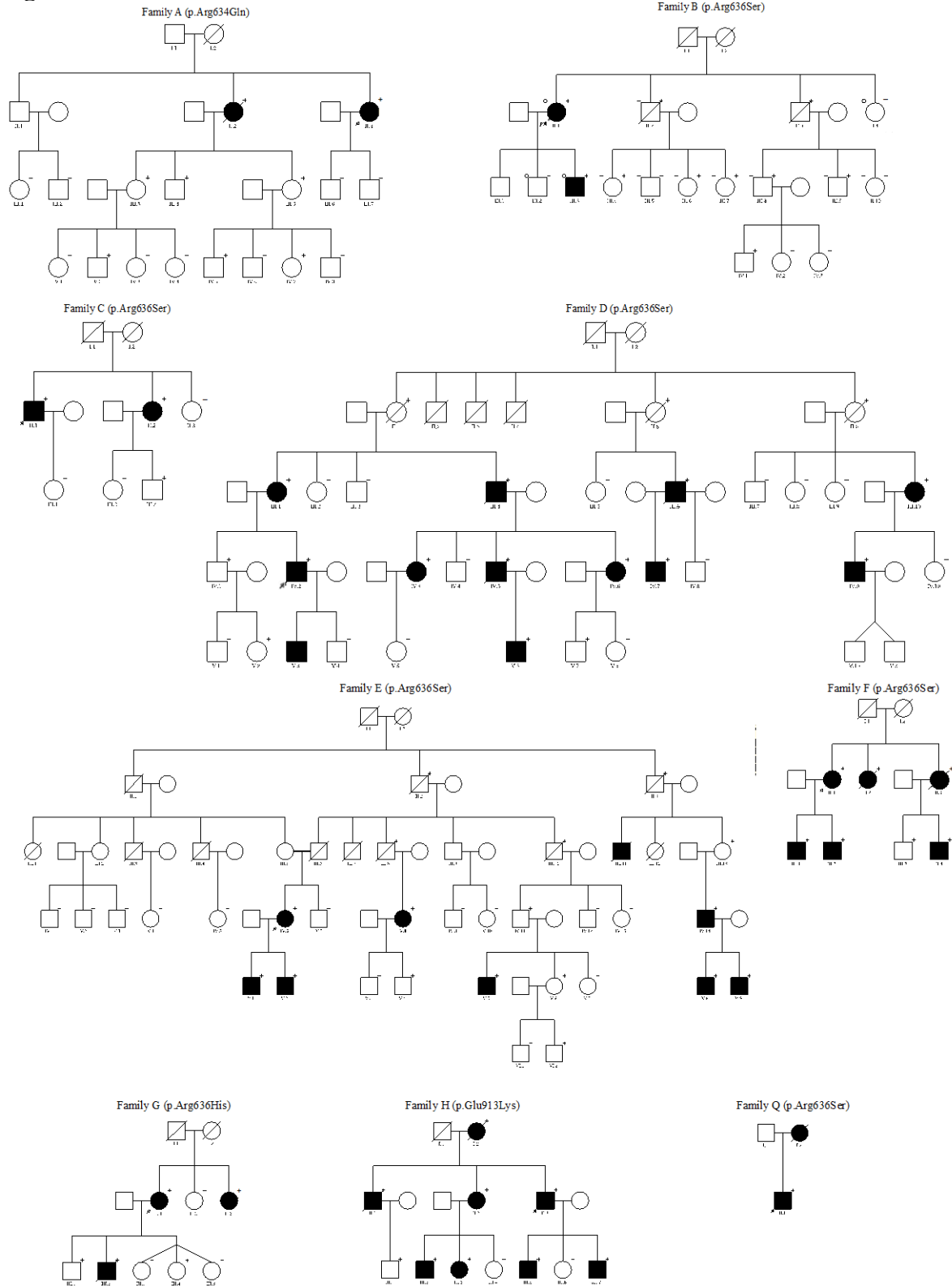
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Figures



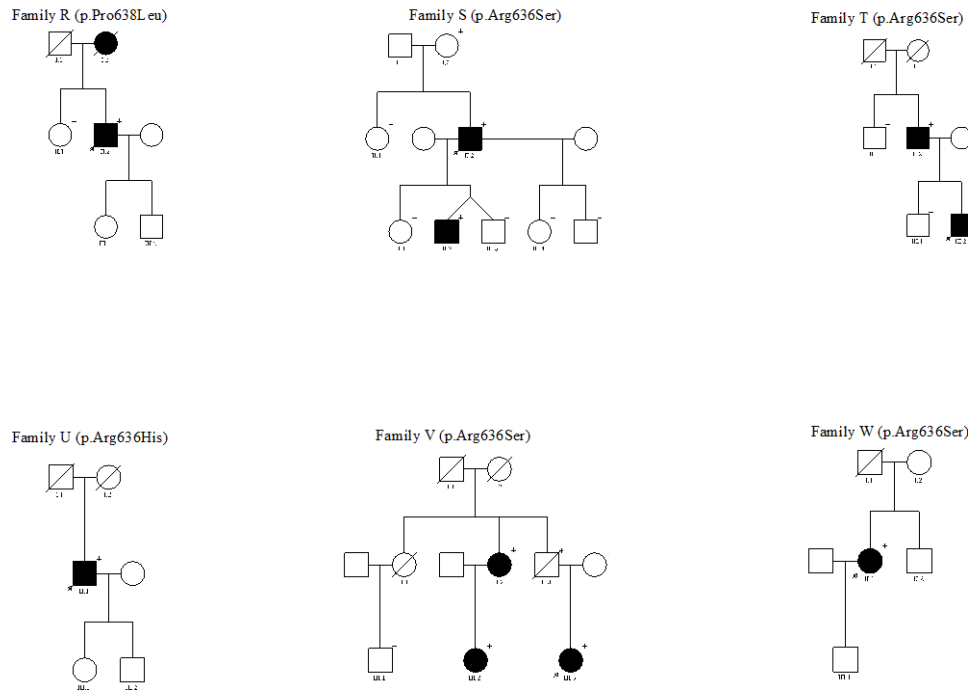


Figure 1 Pedigrees of 15 DCM families carrying pathogenic *RBM20*-variants.

Square = male; circle = female; arrow = index-patient; slash = deceased individual; open symbol = unaffected individual; solid symbol = individual with DCM; plus sign upper right = presence of pathogenic *RBM20*-variant; minus sign upper right = absence of pathogenic *RBM20*-variant; circle upper left = *BAG3* VUS; minus sign upper left = absence of *BAG3* VUS. No clinical data were available in nine obligate *RBM20*-carriers (family D: II.1, II.5, II.6; family E: II.1, II.2, II.3, III.8, III.13; family V: II.3) or in individual II.1 from family B.

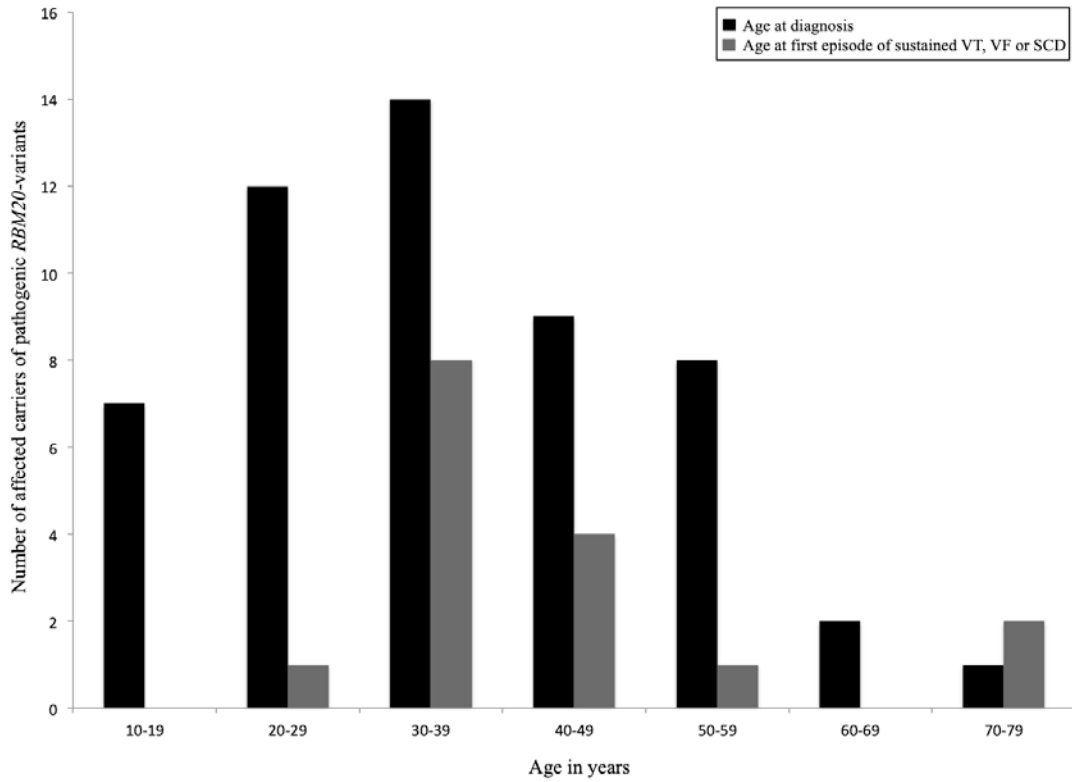


Figure 2

Age distribution among affected carriers of pathogenic *RBM20*-variants at diagnosis in black bars. Grey bars show the age of affected carriers at first episode of ventricular fibrillation, sustained ventricular tachycardia or sudden cardiac death. VF = ventricular fibrillation; VT = ventricular tachycardia.

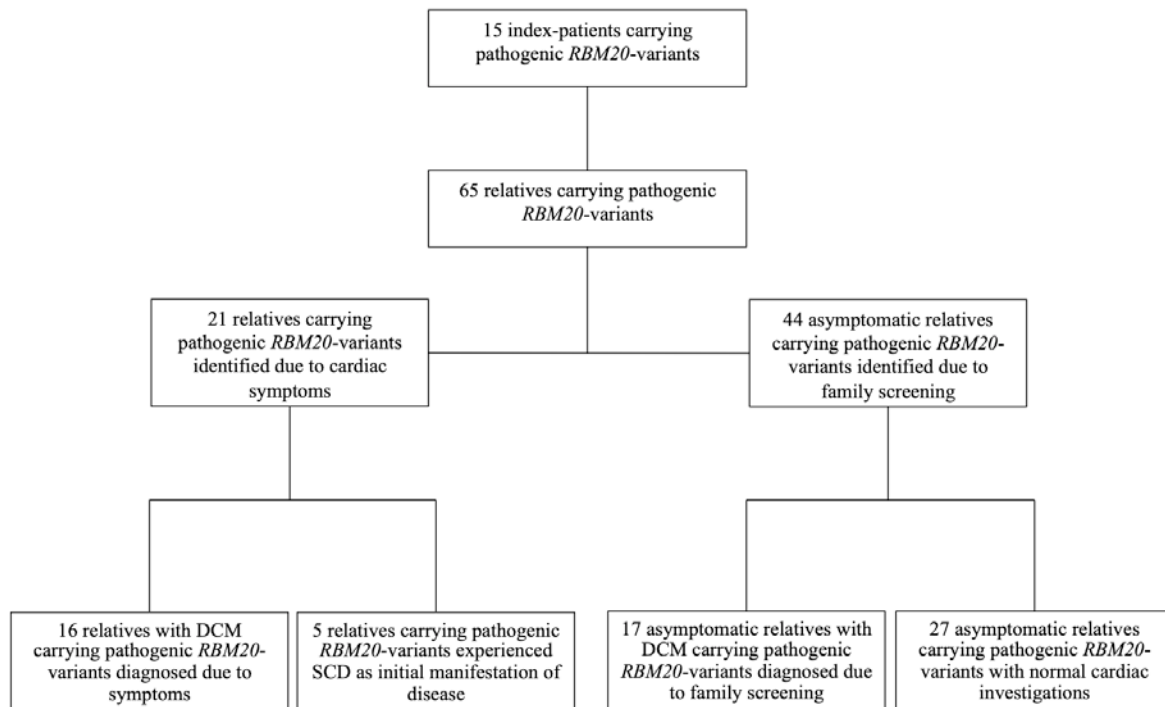


Figure 3

Distribution of carriers of pathogenic *RBM20*-variants identified due to symptoms or by clinical family screening. An additional four index-patients were shown to carry VUS within *RBM20* and did not take part in any of the analyses in the study.

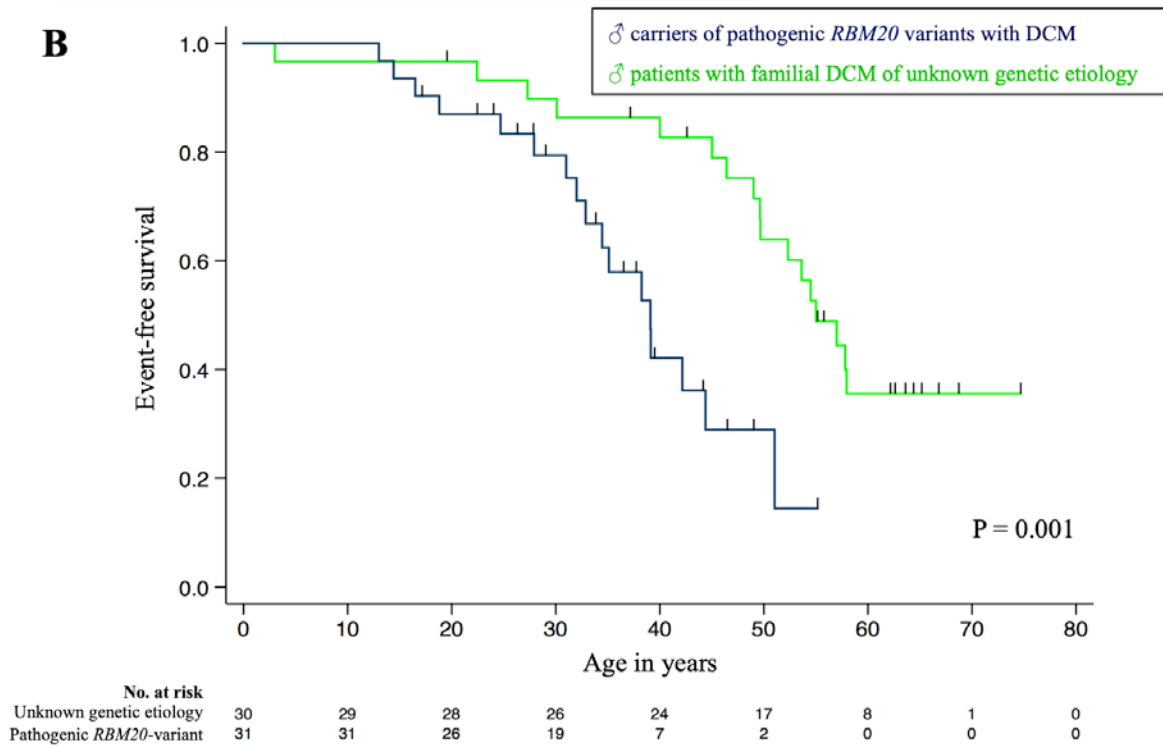
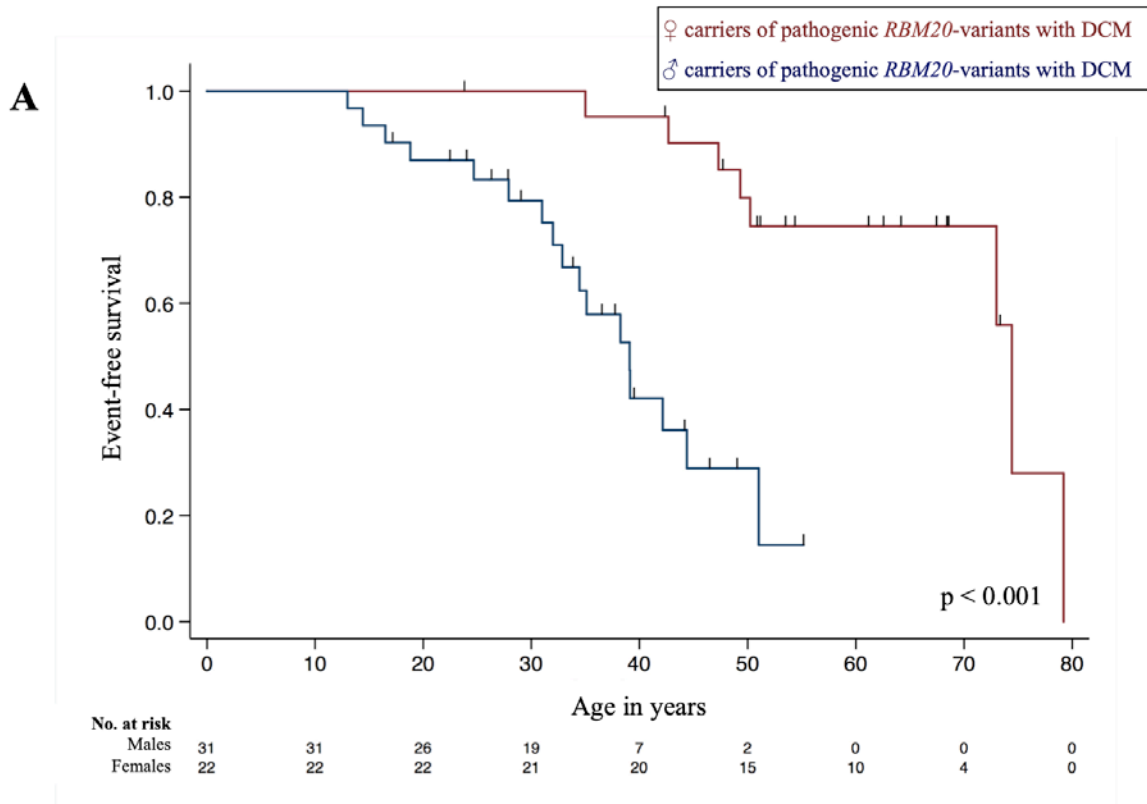


Figure 4

Panel A Event-free survival of males and females with DCM carrying pathogenic *RBM20*-variants. Events were defined as death from end-stage heart failure, cardiac transplantation, sudden cardiac death and first episode of ventricular fibrillation or sustained ventricular tachycardia. Hatch-mark indicates age at most recent follow-up.

Panel B Event-free survival of males with DCM carrying pathogenic *RBM20*-variants and males with familial DCM of unknown genetic etiology. Events were defined as death from end-stage heart failure, cardiac transplantation, sudden cardiac death and first episode of ventricular fibrillation or sustained ventricular tachycardia. Hatch-mark indicates age at most recent follow-up.

Tables

Table 1 Affected Families With DNA-Sequence Variants in *RBM20* and Additional Recognized DCM Genes.

Gene	Predicted amino acid change	Genomic position	Family	Number of affected carriers: current study/previous studies	AF in gnomAD	ACMG criteria	ACMG variant classification
<i>RBM20</i>	p.Arg634Gln/c.1901G>A	112572056	A	2/10 ^{8,10}	-	PS4:PM1;PM2;PP1;PP3	P
<i>RBM20</i>	p.Arg636His/c.1907G>A	112572062	G, U	4/12 ^{6,8,9,10}	-	PS4:PM1;PM2;PP1;PP3	P
<i>RBM20</i>	p.Arg636Ser/c.1906C>A	112572061	B [†] , C, D, E, F, Q, S, T, V, W	38/9 ⁸	-	PS4:PM1;PM2;PP1;PP3	P
<i>RBM20</i>	p.Pro638Leu/c.1913C>T	112572068	R	1/17 ^{8,11}	-	PS4:PM1;PM2;PP1;PP3	P
<i>RBM20</i>	p.Glu913Lys/c.2737G>A	112581114	H	8/0 ¹²	-	PS3;PS4:PM2;PP1;PP3	P
<i>RBM20</i>	p.Met196Val/c.586A>G	112540953	J	2/0	-	PM2;BP4	VUS
<i>ACTC1</i>	p.Ala333Val/c.998C>T	35082749	J	3/0	-	PM2;PP2;PP3	VUS
<i>RBM20</i>	p.Arg392Trp/c.1174C>T	112541541	K	1/0	2/154530	PM2	VUS
<i>TNNI3</i>	p.Lys178Arg/c.530A>G	55665414	K	1/0	-	PM2;PP3	VUS
<i>RBM20</i>	p.Asp674Gly/c.2021A>G	112572176	M	2/0	3/186480	PM2	VUS
<i>RBM20</i>	p.Pro1039Ser/c.3115C>T	112581492	I	3/0	40/188260	-	VUS
<i>TTN</i>	p.Leu20218Tyrfs*118/c.60653delT	179422144	I	2/0	-	PVS1;PM2;PM4	P
<i>BAG3</i>	p.Arg71Trp/c.211C>T	121429393	B [†]	2/0	40/282786	-	VUS

†Affected individuals in family B also carried a rare sequence variant in a different recognized DCM gene (*BAG3*: p.Arg71Trp). ACMG = American College of Medical Genetics and Genomics and the Association for Molecular Pathology. AF = allele frequency; gnomAD = genome aggregation database (<http://gnomad.broadinstitute.org>); P = pathogenic; VUS = variant of unknown significance.

Table 2 Penetrance, Age Distribution and Duration of Follow-up of Individuals Carrying Pathogenic *RBM20*-variants

Pathogenic <i>RBM20</i> -variant	Families/ number of <i>RBM20</i> -carriers	Penetrance: Individuals with DCM/All <i>RBM20</i> -carriers (%)	Age at diagnosis of index-patients ±SD (n)	Age at diagnosis of asymptomatic relatives with DCM identified by cascade screening ±SD (n)	Age at diagnosis of symptomatic relatives with DCM ±SD (n)	Age of individuals who experienced SCD as initial symptom of disease ±SD (n)	Age at most recent follow-up of healthy individuals ±SD (n)	Age at diagnosis of individuals who developed DCM at follow-up ±SD (n)	Median duration of follow-up in months of individuals with DCM* (IQR); (n)
p.Arg634Gln	1/8	2/8 (25)	37 (1)	-	42 (1)	-	35 ± 16 (6)	-	135, 156 (2)
p.Arg636His	2/6	4/6 (67)	27; 46 (2)	44 (1)	14 (1)	-	22; 28 (2)	-	83 (25 – 162); (4)
p.Arg636Ser	10/56	38/56 (68)	45 ± 14 (10)	20 ± 12 (11)	43 ± 14 (13)	37 ± 5 (4)	41 ± 18 (18)	43 (1)	99 (25 – 161); (34)
p.Pro638Leu	1/1	1/1 (100)	13 (1)	-	-	-	-	-	0; (1)
p.Glu913Lys	1/9	8/9 (88)	36 (1)	24 ± 12 (5)	71 (1)	28 (1)	28 (1)	27 ± 15 (3)	37 (11 – 121); (7)
Total	15/80	53/80 (66)	40 ± 15 (15)	29 ± 12 (17)	43 ± 16 (16)	35 ± 6 (5)	38 ± 17 (27)	31 ± 15 (4)	86 (24 – 150); (48)

*Follow-up data was available in 48 individuals since five individuals had died suddenly as the initial manifestation of disease. DCM =

dilated cardiomyopathy; IQR = interquartile range (25-75%); n = number; SCD = sudden cardiac death; SD = standard deviation.

Table 3 Sex Related Disease Expression and Treatment of DCM Patients Carrying Pathogenic *RBM20*-variants

Variables	Total	Male	Female	p
Number of carriers with DCM (%)	53 (100)	31 (58)	22 (42)	0.70
Number of index-patients (%)	15/53 (28)	8/31 (26)	7/22 (32)	0.63
Mean age at diagnosis in years \pm SD	37 \pm 15	29 \pm 11	48 \pm 12	<0.001
Number of individuals with NYHA class \geq II at diagnosis (%)*	26/48 (54)	15/28 (54)	11/20 (55)	0.92
Number of asymptomatic DCM patients identified by family screening (%)	17/53 (32)	12/31 (39)	5/22 (23)	0.22
Age at diagnosis in years of DCM relatives identified by family screening \pm SD	29 \pm 12	26 \pm 12	35 \pm 9	0.20
Mean LVEDd in mm at diagnosis \pm SD [†]	65 \pm 9	68 \pm 9	60 \pm 5	<0.001
Mean LVEDd/BSA in mm/m ² (IQR) [†]	33 (31 – 37)	35 (31 – 38)	32 (31 – 34)	0.21
Mean LVEF in % at diagnosis \pm SD [†]	32 \pm 12	29 \pm 13	38 \pm 9	0.009
Number of individuals treated with an ACE-I or AT-II receptor blocker (%)*	42/48 (88)	24/28 (86)	18/20 (90)	0.69
Number of individuals treated with a beta-blocker (%)*	35/48 (73)	18/28 (64)	17/20 (85)	0.11
Number of individuals who had an ICD implanted (%)*	21/48 (44)	11/28 (39)	10/20 (50)	0.46
Number of individuals who underwent HTx (%)	11/53 (21)	11/31 (34)	-	0.002
Mean age at HTx in years \pm SD	33 \pm 16	33 \pm 16	-	-
Number of individuals who died from end-stage HF (%)	2/53 (4)	-	2/22 (9)	0.17
Age at death in years	54; 73	-	54; 73	-
Number of individuals who experienced SCD, VF or sustained VT (%)	16/53 (30)	9/31 (29)	7/22 (32)	1.00
Mean age at first event of SCD, VF or sustained VT \pm SD	44 \pm 14	36 \pm 5	54 \pm 16	0.006

* Information on NYHA class, medical treatment and ICD implantation was available in 48 individuals. [†] Echocardiography was available in 46 individuals. ACE-I= angiotensin converting enzyme inhibitor; AT-II= angiotensin-II; DCM= dilated cardiomyopathy; HTx= heart transplantation; ICD = implantable cardioverter defibrillator; IQR = interquartile range (25-75%); LVEDd= left ventricular end-diastolic diameter; LVEF= left ventricular ejection fraction; SCD= sudden cardiac death; SD= standard deviation; VF= ventricular fibrillation; VT= ventricular tachycardia.

Table 4 Male Patients With Familial DCM Carrying Pathogenic *RBM20*-variants or variants of Unknown Genetic Etiology

	Total	<i>RBM20</i> carriers	Unknown etiology	p
Number with male sex (%)	61/61 (100)	31/61 (51)	30/61 (49)	-
Number of index-patients (%)	22/61 (36)	8/31 (26)	14/30 (47)	0.10
Mean age in years at diagnosis \pm SD	39 \pm 17	29 \pm 11	49 \pm 16	<0.001
NYHA class \geq II at diagnosis (%)*	31/52 (60)	15/28 (54)	16/24 (67)	0.34
Number of DCM patients identified due to clinical family investigation (%)	17/61 (28)	12/31 (39)	5/30 (17)	0.06
Mean LVEDd at diagnosis in mm \pm SD [†]	68 \pm 10	68 \pm 9	67 \pm 11	0.81
Median LVEDd/BSA at diagnosis in mm/BSA (IQR) [†]	33 (30 – 38)	35 (31 – 38)	32 (30 – 39)	0.69
Mean LVEF at diagnosis in % \pm SD [†]	27 \pm 12	29 \pm 13	28 \pm 12	0.35
Number of individuals treated with ACE-I or AT-II receptor blocker (%)*	42/52 (81)	24/28 (88)	18/24 (78)	0.33
Number of individuals treated with a beta-blocker (%)*	31/52 (60)	18/28 (64)	13/24 (54)	0.45
Number of individuals who had an ICD implanted (%)*	15/52 (29)	11/28 (39)	4/24 (17)	0.07
Number of individuals who received a cardiac transplant (%)	18/61 (30)	11/31 (35)	7/30 (23)	0.30
Mean age in years at HTx \pm SD	38 \pm 16	33 \pm 16	46 \pm 13	0.74
Number of individuals who experienced SCD, VF or sustained VT (%)	17/61 (28)	9/31 (29)	8/27 (30)	0.84
Mean age in years at SCD, VF or sustained VT \pm SD	37 \pm 12	36 \pm 5	38 \pm 18	0.70
Median duration of follow-up in months (IQR)	40 (0 – 98)	48 (4 – 100)	23 (0 – 73)	0.14

*Information about NYHA class, medical treatment was available in 52 individuals.

[†]Echocardiography at diagnosis was available in 50 individuals. ACE-I = angiotensin converting enzyme inhibitor; AT-II = angiotensin-II; HTx = heart transplantation; ICD = implantable cardioverter defibrillator; IQR = interquartile range (25-75%); LVEDd = Left ventricular end-diastolic diameter; LVEF = left ventricular ejection fraction; NYHA= New York Heart Association; SCD = sudden cardiac death; SD = standard deviation; VF = ventricular fibrillation; VT = ventricular tachycardia.

Supplemental Material

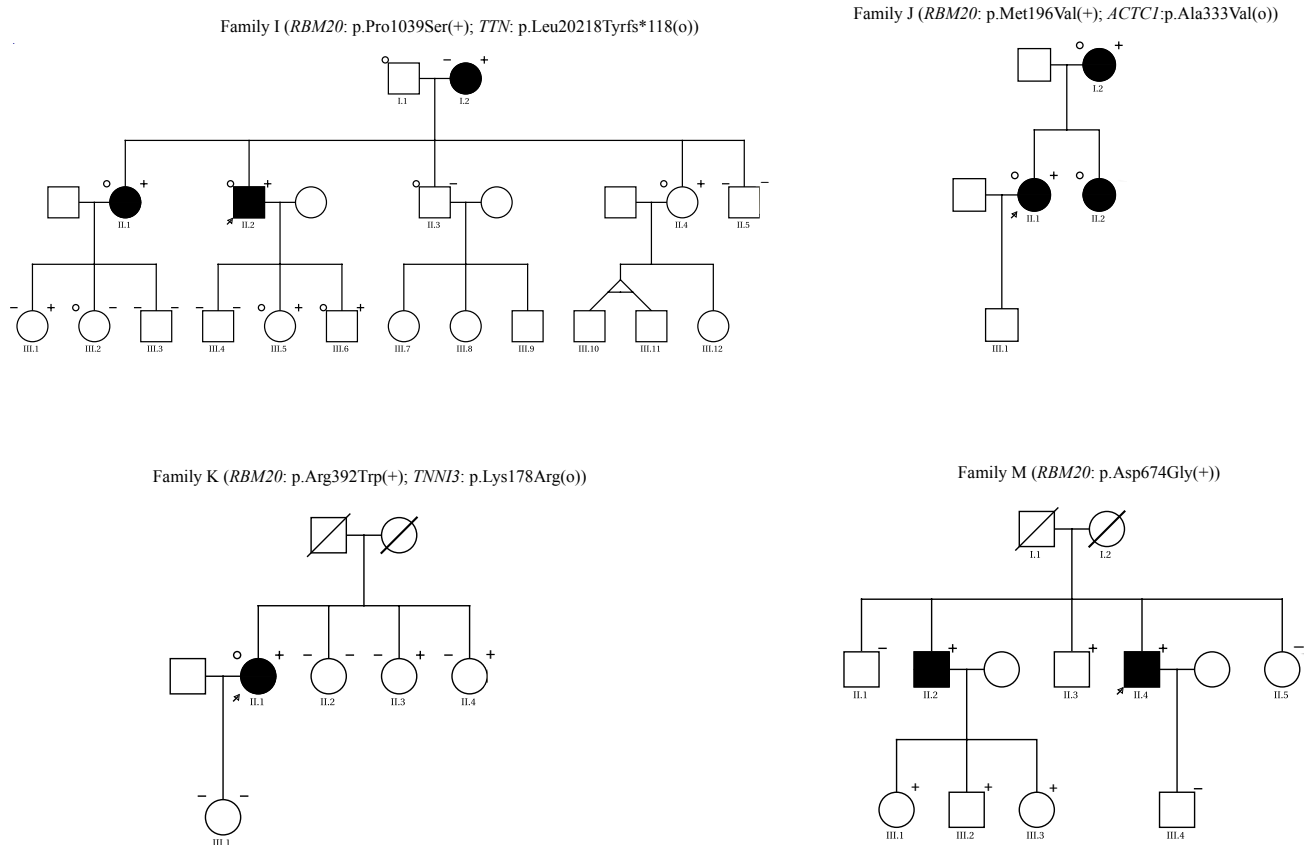


Figure 1S Pedigrees of 4 DCM families carrying DNA-sequence variants of unknown significance (VUS) in *RBM20* and sequence variants in additional recognized DCM genes.

Family I: Individual II.1 and II.2 had DCM and carried a VUS in both *RBM20* and *TTN*. Individual II.4, III.5 and III.6 had a normal cardiac investigation at the age of 48, 22 and 23 respectively. They were shown to carry the same VUS in *RBM20* and *TTN* as their affected relatives. Individual I.2 was diagnosed with DCM at the age of 73 and carried the *RBM20* VUS in isolation. She had also hypertension, type II diabetes and chronic obstructive pulmonary disease which may have explained her cardiac condition.

Family J: Individual I.2 and II.1 had DCM and carried a VUS in both *RBM20* and *ACTC1*. Individual II.2 also had DCM and carried the *ACTC1* VUS while she did not consent to be tested for the VUS in *RBM20*.

Family K: Individual II.1 had DCM and carried a VUS in *RBM20* as well as a VUS in *TNNI3*.

Individual II.3 and II.4 carried the *RBM20* in isolation and had a normal cardiac investigation at the ages of 64 and 67 years, respectively.

Family M: Individual II.2 and II.4 had DCM and carried a VUS in *RBM20*. Individual II.3, III.1, III.2, and III.3 also carried the *RBM20* VUS but had a normal cardiac investigation at the age of 71, 41, 47 and 49 years, respectively.

Square = male; circle = female; arrow = index-patient; Slash = deceased individual; open symbol = unaffected individual; solid symbol = individual with DCM; plus sign upper right = presence of *RBM20* VUS; minus sign upper right = absence of *RBM20* VUS; circle upper left = VUS in additional DCM genes; minus sign upper left = absence of VUS in additional DCM genes.

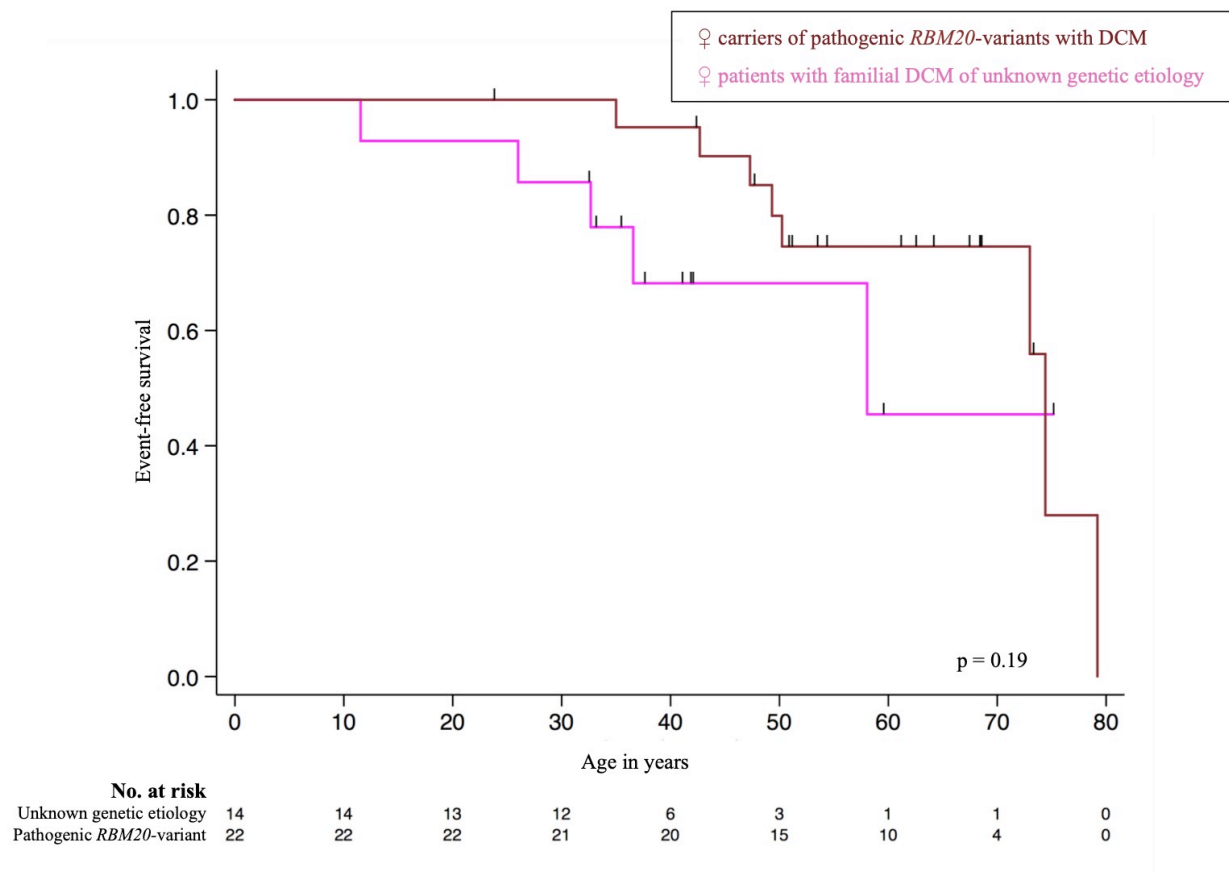


Figure 2S Event-free survival of female carriers of pathogenic *RBM20*-variants with DCM and females with familial DCM of unknown genetic aetiology. Events were defined as death from end-stage heart failure, cardiac transplantation, sudden cardiac death and first episode of ventricular fibrillation or sustained ventricular tachycardia. Hatch-mark indicates age at most recent follow-up.

Table 1S 76 Recognized and Likely DCM Genes Investigated in all Index-patients

Genes
<i>ABCC9, ACTC1, ACTN2, ADRB1, ADRB2, ADRB3, ANKRD1</i>
<i>BAG3</i>
<i>CACNA1C, CACNB2, CALR3, CASQ2, CAV3, CBFEB, CRYAB, CSRP3, CTF1</i>
<i>DES, DMD, DSC2, DSG2, DSP</i>
<i>EYA4</i>
<i>GPD1L</i>
<i>ILK</i>
<i>JPH2, JUNB, JUP</i>
<i>KCNE1, KCNE2, KCNH2, KCNJ2, KCNJ8, KCNQ1, KCNQ2</i>
<i>LAMP2, LDB3, LIMS1, LIMS2, LMNA</i>
<i>MYBPC3, MYH6, MYH7, MYL2, MYL3, MYOZ2, MYPN</i>
<i>NEXN</i>
<i>PARVB, PDLIM3, PKP2, PLN, PRKAG2, PSEN1, PSEN2</i>
<i>RBFOX1, RBM20, RYR2</i>
<i>SCN1B, SCN4B, SCN5A, SGCD, SMYD1, SMYD2, SNTA1</i>
<i>TAZ, TBX20, TCAP, TGFB3, TMPO, TNNC1, TNNI3, TNNT2, TPM1, TTN</i>
<i>VCL</i>

Table 2S Clinical characteristics of Index-patients Carrying pathogenic *RBM20*-variants and their affected relatives at diagnosis and during follow-up.

Variables	Total	Index-patients ^a	Affected relatives who were symptomatic at diagnosis ^b	Affected relatives who were Asymptomatic at diagnosis ^c	p
At diagnosis					
Number of affected <i>RBM20</i> carriers with DCM (%)	53 (100)	15/53 (28)	21/53 (39)	17/53 (32)	-
Mean age at diagnosis ± SD	37 ± 15	40 ± 15	43 ± 16 ^{*c}	29 ± 12 ^{*b}	0.02
Number of males with DCM (%)	31/53 (58)	8/15 (53)	11/21 (52)	12/17 (71)	0.47
Mean LVEDd in mm at diagnosis ± SD ^{†‡}	65 ± 9	64 ± 9	68 ± 11	62 ± 5	0.12
Mean LVEDd/BSA at diagnosis in mm/m ² (IQR) ^{†‡}	33 (31 – 37)	32 (31 – 36)	36 (32 – 39)	32 (31 – 35)	0.10
Mean LVEF in % at diagnosis ± SD ^{†‡}	32 ± 12	32 ± 13	27 ± 12 ^{*c}	38 ± 8 ^{*b}	0.03
At follow-up					
Number of individuals treated with an ACE-I/AT-II (%) [‡]	42/48 (88)	14/15 (93)	14/16 (88)	14/17 (82)	0.65
Number of individuals treated with a beta-blocker (%) [‡]	35/48 (73)	13/15 (87)	12/16 (75)	10/17 (59)	0.20
Number of individuals who had an ICD implanted (%) [‡]	21/48 (44)	6/15 (40)	9/16 (38)	6/17 (35)	0.45
Number of individuals who underwent HTx (%)	11/53 (21)	5/15 (33)	4/21 (19)	2/17 (12)	0.31
Mean age at HTx ± SD	33 ± 16	39 ± 17	32 ± 15	17; 19	0.30
Number of individuals who died from end-stage HF (%)	2/53 (4)	-	2/16 (13)	-	-
Mean age of death ± SD	54; 73		54; 73	-	-
Number of individuals who experienced a VA (%)	16/53 (30)	4/15 (27)	11/21 (52) ^{*c}	1/17 (6) ^{*b}	0.008
Mean age at first event of VA ±SD	44 ± 14	45 ± 5	44 ± 17	35	0.84
Median duration of follow-up in months (IQR)	86 (24 – 150)	117 (18 – 163)	99 (40 – 195)	48 (11 – 127)	0.32

*Statistical significant difference between groups (^a = Index-patients, ^b = Affected relatives who were symptomatic at diagnosis and ^c = Affected relatives who were asymptomatic at diagnosis).

[†]Echocardiography was available in 46 individuals. [‡]Five relatives who died suddenly as initial manifestation of disease were not included. ACE-I= angiotensin converting enzyme inhibitor; AT-

II= angiotensin-II; DCM= dilated cardiomyopathy; HTx= heart transplantation; ICD = implantable cardioverter defibrillator; IQR = interquartile range (25-75%); LVEDd= left ventricular end-diastolic diameter; LVEF= left ventricular ejection fraction; SD= standard deviation; VA= ventricular arrhythmia (including ventricular fibrillation, sustained ventricular tachycardia and sudden cardiac death).

Table 3S Female Patients with Familial DCM Caused by Pathogenic *RBM20*-variants or of Unknown Genetic Etiology

	Total	<i>RBM20</i> carrier	Unknown etiology	p
Number with female sex (%)	36/36 (100)	22/36 (51)	14/36 (49)	-
Number of index-patients (%)	14/36 (39)	7/22 (32)	7/14 (50)	0.28
Mean age in years at diagnosis \pm SD	42 \pm 15	48 \pm 12	33 \pm 16	0.003
NYHA class \geq II at diagnosis (%) [*]	18/33 (55)	11/20 (55)	6/13 (46)	0.62
Number of DCM patients identified due to clinical family investigation (%)	9/36 (25)	5/22 (23)	4/14 (29)	0.69
Mean LVEDd at diagnosis in mm \pm SD [†]	60 \pm 6	60 \pm 5	61 \pm 8	0.51
Median LVEDd/BSA at diagnosis in mm/m ² (IQR) [†]	33 (31 – 36)	32 (31 – 34)	36 (30 – 39)	0.12
Mean LVEF at diagnosis in % \pm SD [†]	36 \pm 10	38 \pm 9	32 \pm 11	0.11
Number of individuals treated with a ACE-I or AT-II receptor blocker (%) [*]	29/33 (83)	18/20 (90)	11/13 (85)	0.64
Number of individuals treated with a beta-blocker (%) [*]	25/33 (76)	17/20 (85)	8/13 (62)	0.12
Number of individuals who had an ICD implanted (%) [*]	13/33 (39)	10/20 (50)	3/13 (23)	0.12
Number of individuals who received a cardiac transplant (%)	3/36 (8)	-	3/14 (21)	0.05
Age in years at HTx	12; 37; 42	-	12; 37; 42	-
Number of individuals who experienced SCD, VF or sustained VT (%)	9/36 (25)	7/22 (32)	2/14 (14)	0.43
Mean age in years at SCD, VF or sustained VT \pm SD	52 \pm 16	54 \pm 16	32; 58	-
Median duration of follow-up in months (IQR)	116 (48 – 186)	135 (55 – 191)	69 (43 – 115)	0.12

^{*}Information about NYHA class, medical treatment and ICD implantation was available in 33 individuals. [†]Echocardiography at diagnosis was available in 33 individuals. ACE-I = angiotensin converting enzyme inhibitor; AT-II = angiotensin-II; HTx = heart transplantation; ICD = implantable cardioverter defibrillator; IQR = interquartile range (25-75%); LVEDd = Left ventricular end-diastolic diameter; LVEF = left ventricular ejection fraction; NYHA = New York Heart Association; SCD = sudden cardiac death; SD = standard deviation; VF = ventricular fibrillation; VT = ventricular tachycardia.