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Title	Filamin C variants are associated with a distinctive clinical and immunohistochemical arrhythmogenic cardiomyopathy phenotype
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Abstract

Background: Pathogenic variants in the filamin C (FLNC) gene are associated with inherited cardiomyopathies including dilated cardiomyopathy with an arrhythmogenic phenotype. We evaluated FLNC variants in arrhythmogenic cardiomyopathy (ACM) and investigated the disease mechanism at a molecular level. Methods: 120 gene-elusive ACM patients who fulfilled diagnostic criteria for arrhythmogenic right ventricular cardiomyopathy (ARVC) were screened by whole exome sequencing. Fixed cardiac tissue from FLNC variant carriers who had died suddenly was investigated by histology and immunohistochemistry. Results: Novel or rare FLNC variants, four null and five variants of unknown significance, were identified in nine ACM probands (7.5%). In FLNC null variant carriers (including family members, n=16) Task Force diagnostic electrocardiogram repolarization/depolarization abnormalities were uncommon (19%), echocardiography was normal in 69%, while 56% had >500 ventricular ectopics/24 hours or ventricular tachycardia on Holter and 67% had late gadolinium enhancement (LGE) on cardiac magnetic resonance imaging (CMRI). Ten gene positive individuals (63%) had abnormalities on ECG or CMRI that are not included in the current diagnostic criteria for ARVC. Immunohistochemistry showed altered key protein distribution, distinctive from that observed in ARVC, predominantly in the left ventricle. Conclusions: ACM associated with FLNC variants presents with a distinctive phenotype characterized by Holter arrhythmia and LGE on CMRI with unremarkable ECG and echocardiographic findings. Clinical presentation in asymptomatic mutation carriers at risk of sudden death may include abnormalities which are currently non-diagnostic for ARVC. At the molecular level, the pathogenic mechanism related to FLNC appears different to classic forms of ARVC caused by desmosomal mutations.

Keywords	Arrhythmogenic cardiomyopathy; ARVC; filamin C variants; immunohistochemistry; late gadolinium enhancement
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Highlights

- *FLNC*-linked ACM presents with LV involvement, ventricular arrhythmia, sudden death
- Late gadolinium enhancement on cardiac MRI may be the only structural abnormality
- Mutation carriers have abnormalities on ECG or cardiac MRI non-diagnostic for ARVC
- *FLNC* disease mechanism different to classic ARVC caused by desmosomal mutations

ABSTRACT

Background: Pathogenic variants in the filamin C (*FLNC)* gene are associated with inherited cardiomyopathies including dilated cardiomyopathy with an arrhythmogenic phenotype. We evaluated *FLNC* variants in arrhythmogenic cardiomyopathy (ACM) and investigated the disease mechanism at a molecular level.

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Conclusions: ACM associated with *FLNC* variants presents with a distinctive phenotype characterized by Holter arrhythmia and LGE on CMRI with unremarkable ECG and echocardiographic findings. Clinical presentation in asymptomatic mutation carriers at risk of sudden death may include abnormalities which are currently non-diagnostic for ARVC. At the molecular level, the pathogenic mechanism related to *FLNC* appears different to classic forms of ARVC caused by desmosomal mutations.

Filamin C variants are associated with a distinctive clinical and immunohistochemical arrhythmogenic cardiomyopathy phenotype

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Keywords: Arrhythmogenic cardiomyopathy; ARVC; filamin C variants; immunohistochemistry; late gadolinium enhancement

ABSTRACT

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Methods: 120 gene-elusive ACM patients who fulfilled diagnostic criteria for arrhythmogenic right ventricular cardiomyopathy (ARVC) were screened by whole exome sequencing. Fixed cardiac tissue from *FLNC* variant carriers who had died suddenly was investigated by histology and immunohistochemistry.

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1. Introduction

Efforts to improve early detection of individuals at risk of life threatening arrhythmia from inherited cardiovascular disease are ongoing [1]. Identification of patients who present with arrhythmias independent of or not explained by recognised causes of cardiac disease has led to the proposal for the term arrhythmogenic cardiomyopathy (ACM) [2-4]. The recognition of a number of inherited arrhythmogenic cardiomyopathies has led to the recent broader acceptance of this term [1]. Incorporated within this classification are patients who present with ventricular arrhythmia in association with right, left or biventricular disease. Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) caused by mutations in desmosomal genes is the most studied of the ACMs with well established clinical and pathological diagnostic criteria [5, 6].

ACM with prominent left ventricle involvement can be indistinguishable from arrhythmogenic forms of dilated cardiomyopathy (DCM) both clinically and genetically [7]. The *FLNC* gene, encoding the major cardiac structural protein filamin C, has been implicated in inherited forms of cardiomyopathy, including DCM [8]. Recent studies have reported truncating variants in *FLNC* as the cause of DCM with life-threatening ventricular arrhythmia [9, 10]. Clinical evaluation of *FLNC* mutation carriers and molecular characterization of cardiac tissue from a patient carrying a truncating variant have shown a link between DCM and ARVC, both at the clinical and cellular level [10]. However, to date, the causal role of *FLNC* variants in ACM cases fulfilling Task Force diagnostic criteria for ARVC has not been directly investigated. As a result, the clinical and molecular phenotype of *FLNC*-associated ACM is currently unknown.

In this study, we investigate the clinical characteristics of *FLNC* mutation carriers in ACM pedigrees in which the index cases fulfil current diagnostic criteria for ARVC. We also report

the distribution of key proteins in myocardial tissue with *FLNC* variants and compare it with that observed in classic forms of the disorder.

2. Methods

2.1 Patient cohort

Genetic screening of 269 ACM patients by targeted next generation sequencing as described by Lopes et al [11] identified a group of 120 index cases who were free of potentially pathogenic variants in major genes associated with cardiomyopathy, arrhythmia and heart failure syndromes. This gene-elusive cohort underwent whole exome sequencing in order to identify novel causative ACM genes.

Patients were referred to the Inherited Cardiovascular Disease Unit at the Heart Hospital (prior to 2014) and the Barts Cardiac Centre, St Bartholomew's Hospital with a suspicion of ACM or with a premature sudden cardiac death and/or known ACM in their family. Clinical diagnosis of arrhythmogenic cardiomyopathy was based on the Task Force diagnostic criteria [6]. Index cases included in this study had a diagnosis of definite or borderline ARVC based on the fulfilment of the current Task Force diagnostic criteria for the disorder [6], or had a possible diagnosis on account of a first-degree relative who died suddenly aged less than 35 years with a post mortem (PM) diagnosis of ARVC. Detailed clinical evaluation included medical and family history, 12-lead electrocardiogram (ECG), signal averaged ECG (SAECG), 24-hour ambulatory ECG, standard 2D transthoracic echocardiogram and cardiac magnetic resonance imaging (CMRI). When appropriate clinical phenotyping and genetic testing was offered to extended relatives within pedigrees.

This study conforms with the ethical guidelines of the 1975 Declaration of Helsinki and has received approval by the National Health Service (NHS) Ethics Committees (REC ID: 15/LO/0549, UK) and CEIC Hospital Virgen de la Arrixaca (Spain) and CEIC Hospital

Universitario y Politécnico La Fe (Spain). Informed written consent for inclusion in the study was obtained from all participants or, in cases of minors or deceased individuals, from first-degree family members.

2.2 Whole exome sequencing (WES)

In this study we performed whole exome sequencing on DNA samples from ACM index cases and family members. DNA from whole blood, paraffin-embedded cardiac tissue and saliva samples was extracted utilizing kits and protocols from Qiagen and DNA Genotek. Genomic DNA was subjected to sample preparation as per the protocol recommended by Agilent for the SureSelect^{XT} Target Enrichment for Illumina paired-end multiplexed sequencing method. Targeted exonic regions were captured using the Agilent SureSelect^{XT} Human Exon V5 following the manufacturer's protocol. Enriched DNA libraries were exome sequenced on the Illumina NextSeq500 platform as paired-end 75 base reads at a minimum of 30x coverage. Bioinformatic analysis of WES data including copy number variation was based on an in-house developed pipeline (Supplementary material, Appendix).

2.3 Immunohistochemistry

As cardiac tissue was not available from British patients screened by WES, in order to determine the effects of *FLNC* mutations at the level of the intercalated disc, post mortem cardiac tissue from patients with FLNC variants was sourced from a Spanish clinicopathology consortium. Right and left ventricle (RV and LV) specimens from eleven sudden cardiac death (SCD) victims with a diagnosis of arrhythmogenic cardiomyopathy carrying potentially pathogenic *FLNC* variants were included in this study. Post-mortem (PM) examination protocol was in keeping with published guidelines. The patients, all male, (aged 16–52, mean age of death 33.5 years) had a diagnosis of ARVC or left dominant arrhythmogenic cardiomyopathy at autopsy due to the presence of fibrosis and fat infiltration (nine cases) and

predominant fibrosis (3 cases). Cardiac samples from those cases were fixed in formalin and preserved in paraffin blocks. Immunohistochemical analysis of key proteins previously implicated in the molecular pathogenesis of classical ARVC in myocardial tissue was carried out based on the protocol developed by Asimaki et al [12]. Detailed description of the method is provided in the Appendix. Tissue samples from age-matched individuals with no clinical or pathological evidence of heart disease were subjected to the same protocol and used as negative controls (n=5). In summary, RV and LV specimens from each SCD case were stained for filamin C, plakoglobin, desmoplakin, connexin 43, synapse-associated protein SAP97 and glycogen synthase kinase 3β, GSK3β.

3. Results

3.1 FLNC variants

WES of a cohort of 120 gene-elusive ACM index cases identified seven novel and two rare *FLNC* variants (7.5%). They include four null variants (three nonsense and one splice site variant) which are predicted to be pathogenic based on the American College of Medical Genetics and Genomics (ACMG) guidelines and five variants of unknown significance (VUS, one in-frame deletion and four missense variants) [13]. Details of these variants are given in Appendix Table A1. *FLNC* variants identified by WES were confirmed by Sanger DNA sequencing. There were no *FLNC* copy number variants identified in the cohort.

3.2 Clinical phenotypes of FLNC variant carriers

Index cases carrying *FLNC* variants had a diagnosis of definite (n=4), borderline (n=2) or possible (n=3) ARVC based on the Task Force diagnostic criteria [6]. All had at least one

sudden cardiac death victim in their extended families ranging from 20 to 71 years (median 40yrs) and ARVC or arrhythmogenic left ventricular cardiomyopathy (ALVC) was diagnosed at PM in six deceased family members (Appendix Table A2).

Segregation analysis and cascade genetic screening with clinical evaluation of relatives in pedigrees was feasible in the four families carrying the null *FLNC* variants (Figure 1). Detailed clinical features for *FLNC* variant carriers in Families A-D are provided in Table 1. For the VUS variants, pedigree analysis was possible in only two cases (Families E and F) which carried the p.59_62DLQRdel and p.K2260R variants respectively (Appendix Figure A1). No family members of index cases G, H and I were available. Clinical characteristics of *FLNC* VUS carriers are given in Appendix Table A3. There was no evidence of skeletal muscle abnormalities in ACM index cases or their relatives and serum creatine kinase levels were normal in those tested. In addition to ACM index cases, another 26 relatives were clinically evaluated and genotyped for *FLNC* variants; fourteen of them were genotype positive. DNA from two SCD cases was available and those individuals were also found to be *FLNC* variants carriers (Figures 1 and A1 and Tables 1 and A3).

Due to the limited clinical information on pedigrees with *FLNC* VUS variants and the ambiguity regarding possible pathogenicity of such variants, analysis focused on the ACM families with *FLNC* null variants (index cases and relatives, n=16). In this cohort the presence of Task Force diagnostic ECG repolarization and depolarization abnormalities were uncommon, n=2 (12.5%) and n=1 (6.25%) respectively. ECG was unremarkable in 5 genotype positive individuals (31%), abnormalities in the remaining included low voltage 5/16 (32%) and poor R wave progression across anterior chest leads 1/16 (6%). In the majority of cases echocardiogram did not reveal overt abnormalities (n=11, 69%). Clinical presentation with palpitation and/or syncope was uncommon, however, 7 patients (44%) had non sustained VT and/or >500 VES/24 hours and 2 (12.5%) presented with sustained VT. Of 15 *FLNC* null

variant carriers who had cardiac MRI, the majority (n=10, 67%) showed late gadolinium enhancement (LGE). Characteristic ECG and CMR images from a *FLNC* variant carrier are shown in Figure 2.

3.3 Immunohistochemical analysis of fixed myocardial tissue

Histological examination of eleven cardiac specimens from sudden death victims (numbered 1 to 11, Appendix Table A4) carrying *FLNC* mutations was performed at post mortem. Mean weight was 471.3 ± 58.9 g. All but two cases had normal internal left ventricular measurements (mean LV diameter 37.4 ± 11.0 mm). Wall thickness was within normal limits in all hearts (11-13 mm).

Evidence of fibrosis was present in both ventricles in ten samples; fibrofatty replacement was evident in three samples whilst four specimens showed signs of fibrosis and inflammation, mainly in the left ventricle. Distribution of fibrofatty infiltration in the left ventricle was circumferential in 7 (mesocardial in 4 and subepicardial in 3) and inferolateral subepicardial in 4 cases. Infiltration which was predominant in the inferolateral wall, extended from the basal to the apical segments. Inflammatory infiltrates affecting the left ventricle were multifocal in 2 and extensive in another 2. There was only one specimen (case no 9) with remarkable inflammatory infiltrates in the right ventricle, which also had extensive left involvement. The same sample had no evidence of fibrosis at PM, however, fat infiltration was present. Characteristic histology images are shown in Appendix Figure A2.

Cardiac specimens used for immunohistochemistry experiments originated from ACM patients with *FLNC* variants who had suffered sudden cardiac death. Those included three deletions and eight single nucleotide substitutions (two splice site, four termination and two

missense variants). Two unrelated cases carried the same nonsense mutation: c.5398G>T; p.G1800X. A list of *FLNC* variants in fixed tissue samples is given in Appendix Table A4.

Immunoreactive signal for filamin C was strong and indistinguishable from controls in RV samples from ten ACM cases but it was found to be reduced in LV specimens from all eleven cases. The signal for plakoglobin was strong and indistinguishable from controls in nine cases in both RV and LV samples. Junctional signal for Cx43 was reduced in two RV samples and six LV samples. Moreover, signal for the desmosomal protein desmoplakin was found to be reduced at cell-cell junctions in eight RV samples and five LV samples. In contrast, GSK3β was present in the cytosol in all *FLNC* cases examined as in control myocardium samples. Finally, immunoreactive signal for SAP97 appeared reduced in the majority of RV and LV samples whilst in two cases this protein was only detected in the sarcomere. Characteristic confocal microscopy images from case no 6 are displayed in Figure 3. Immunohistochemistry data from RV and LV specimens from all eleven cases are summarised in Appendix Figure A3.

4. Discussion

For the first time we performed clinical characterization of *FLNC* variants in an ACM cohort; previous studies have focused on pure DCM or DCM with an arrhythmogenic component [8-10]. We observed marked phenotypic differences in ACM associated with *FLNC* null variants compared to classic ARVC caused by desmosomal gene mutations.

Repolarization (e.g. T wave inversion) and depolarization (such as prolonged terminal activation duration and epsilon waves) abnormalities are considered typical diagnostic features for classic forms of ARVC [6]. However, in our *FLNC* cohort only three patients (19%) had Task Force diagnostic ECG repolarization and depolarization abnormalities. In contrast, the majority of *FLNC* null variant carriers (69%) had ECG repolarization and depolarization

abnormalities, such as right bundle branch block (RBBB) and loss of inferior R waves, which, in isolation, are not considered diagnostic criteria [6]. Similarly, standard echocardiographic imaging has been shown to detect structural abnormalities in the majority of ARVC patients [14] but 69% of our cases had no detectable echocardiographic disease features. Moreover, the most striking observation was that MRI showed the presence of LGE with preserved ventricular function in all index cases and the majority of gene positive family members, a clinical feature which is not currently a diagnostic criterion for ARVC [6]. Consequently, in these cases, strict adherence to the Task Force diagnostic criteria, can lead to individuals at risk being incorrectly classified as either unaffected or being at low risk of complications. This highlights the importance of genetic evaluation of asymptomatic family members and the need of more detailed phenotyping targeting recognized features of particular subtypes of ACM. In this study familial evaluation limited to ECG and echocardiography would not have identified the majority of at-risk individuals whereas significant abnormalities were detected with ECG monitoring and CMRI, for example individuals IV:1 (Family A) and II:1 and III:4 (Family C).

In summary, we present the clinical phenotype of *FLNC* families with index cases who fulfil Task Force diagnostic criteria for ARVC. It is characterized by predominant LV involvement; frequently non-diagnostic electrocardiography and echocardiography; frequent ventricular ectopy or non-sustained VT on 24-hour Holter monitoring and fibrosis (late gadolinium enhancement) on MRI. Notably there is a high incidence of adverse cardiovascular events, highlighted in our cohort with a family history of multiple sudden cardiac death victims at a young age. This clinical pattern appears similar to emerging experience of other genetically determined arrhythmogenic cardiomyopathies caused by mutations in TMEM43, phospholamban, desmin and lamin A/C [15-18]. All may present with life threatening arrhythmia, myocardial structural abnormalities, usually predominantly of the left ventricle, though patients have been reported who fulfil Task Force diagnostic criteria. This highlights

the need for evolution of the current classification with use of the term 'arrhythmogenic cardiomyopathy' which incorporates ARVC as well as other inherited and acquired forms of ACM. The recent Heart Rhythm Society guidelines for the diagnosis and management of arrhythmogenic cardiomyopathy recognize this evolving scenario [1].

Previous studies have highlighted the importance of immunohistochemistry of cardiac tissue from mutation carriers in investigating the disease mechanisms related to ACM [19, 20]. In this study, we sought to characterize the molecular profile of fixed RV and LV specimens from ACM sudden death cases. It has been previously shown that filamin C displays a strong localisation at the intercalated disc that decreases or is completely absent in patients with restrictive and dilated cardiomyopathy carrying *FLNC* mutations [8, 21]. Here, staining for FLNC showed a decreased immunoreactive signal intensity in the left ventricle in all eleven ACM cases highlighting a predominant left ventricle disease pattern associated with mutations in this gene. This is consistent with a recent study that reported reduced immunohistochemical staining signal for filamin C in left ventricle samples from an arrhythmogenic DCM patient who carried the p.G1891Vfs61X mutation [10].

It is now well established that in ARVC plakoglobin translocates from the intercalated discs to intracellular pools [19], an observation that is considered as a "hallmark" of disease pathogenesis [22]. However, the signal for plakoglobin was strong and indistinguishable from controls in nine cases (82%) examined in our *FLNC* cohort in both left and right ventricular samples. Interestingly, signal for plakoglobin was decreased in both RV and LV specimens carrying the two missense variants (p.K35N and p.T160K). However, at present, the significance of this finding is unclear. Similar to plakoglobin mislocalization, the enzyme GSK3β is re-distributed from the cytosol to the intercalated disc in classic ARVC [23]. However, none of the eleven *FLNC* cases examined showed this re-distribution.

Immunoreactive signal for the major gap junction protein Cx43 is usually significantly depressed at cardiac intercalated discs in patients with ARVC [23]. In our *FLNC* cohort, junctional signal for Cx43 was reduced only in two (20%) RV and six LV (60%) cases. Considering the advanced disease state and predominant LV involvement in all our *FLNC* cases, it is unclear whether Cx43 remodelling played a primary role in ACM pathogenesis or was a result of the histological changes in the myocardium. The signal for the desmosomal protein desmoplakin was found to be reduced at cell-cell junctions mainly in RV samples (80%) and when DSP staining intensity was reduced in the left ventricle, the corresponding RV signal for the same case was also reduced. Altered desmoplakin localisation has been reported in left dominant arrhythmogenic cardiomyopathy [24] and, as our *FLNC* cases had a predominantly left dominant pattern of disease, the observed reduction of desmoplakin signal is in line with this phenotype.

SAP97 is a membrane-associated guanylate kinase reported to show consistently decreased immunohistochemistry staining intensity in both the sarcomeric and junctional pools in the myocardium of desmosomal ACM patients independently of the specific causative mutation [25]. In our cohort SAP97 was reduced in the majority of RV and LV specimens whilst in two cases SAP97 signal was detectable in the sarcomeres but not at the intercalated discs. This finding is consistent with previous reports on myocardial samples from patients with end-stage ischemic, dilated and hypertrophic cardiomyopathy [25].

Overall, we observed a specific localization pattern in our cohort for three proteins: FLNC signal was reduced in all cases; plakoglobin signal was normal in the large majority of RV and LV specimens and GSK3β signal was normal in all cases tested. All these findings point to a disease pattern different to classic ARVC and are consistent with the hypothesis that ACM associated with *FLNC* variants presents with a left dominant arrhythmogenic cardiomyopathy phenotype which may manifest via a different mechanism to typical right ventricular

arrhythmogenic cardiomyopathy. Begay et al 2018 observed similar immunohistochemistry results to our data for a patient with the p.G1891Vfs61X *FLNC* mutation, namely normal plakoglobin immunostaining signal, typical GSK3β cytoplasmic distribution and reduced DSP signal compared to control samples [10].

Collectively, our analysis suggests that the clinical and molecular "signature' of *FLNC* cardiomyopathy is distinct to that of ARVC. These results indicate that these two clinical entities reflect different molecular mechanisms of pathogenesis.

4.1 Limitations

This study is limited by the small number of recruited *FLNC* variant carriers which, in part, is attributed to the low frequency of causative *FLNC* variants in ACM and the high genetic heterogeneity that characterizes the disorder. As it is common in studies of cardiomyopathy patients, small family sizes have restricted our ability to perform extensive segregation analysis in all *FLNC* cases.

Immunohistochemical analysis relied on the availability of paraffin fixed tissue. The challenges in collecting human heart samples are well known. Therefore, this study was also hindered by limited quantity of tissue for each case and some samples were not immunostained for a complete set of proteins.

5. Conclusion

ACM related to *FLNC* variants presents with a distinctive phenotype that may not be recognized by current Task Force ARVC diagnostic criteria or by familial evaluation limited to ECG or echocardiography. Physicians should be aware of "non-diagnostic" disease features in asymptomatic gene positive individuals.

The molecular mechanism of pathogenesis of this form of ACM is markedly different to classic ARVC and does not involve mislocalization of plakoglobin or GSK3β.

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Figure legends

Fig. 1. Pedigrees of ACM families with *FLNC* variants.

Squares indicate males; circles, females; slashes, deceased individuals; black symbols, definite diagnosis of ARVC based on current Task Force diagnostic criteria [6] or evidence of ARVC at post mortem; grey symbols, borderline diagnosis of ARVC; hatched symbols, gene positive individuals with possible diagnosis of ARVC; (+), positive genotype for *FLNC* variant; (-), negative genotype for *FLNC* variant; Arrows indicate the index case in each family. ALVC, arrhythmogenic left ventricular cardiomyopathy; PM, post mortem; SCD, sudden cardiac death. Gene negative individuals with a possible diagnosis of ARVC based solely on family history are depicted as unaffected.

Fig. 2. ECG and cardiac MR images from individual IV:1 (Family A) who was clinically screened due to family history of ACM.

A). Electrocardiogram showing inferior lead T-wave inversion (III and aVF).

B). CMRI two-chamber view of the left ventricle (left image) and short axis view (right image) showing basal lateral subepicardial late gadolinium enhancement (white arrows).

Fig. 3. Immunohistochemistry staining of paraffin-embedded cardiac specimens from case no 6 carrying the p.Y705X *FLNC* variant.

Top panel, control sample; middle panel, RV sample; bottom panel, LV sample. Immunoreactive signal for plakoglobin and GSK3β at the intercalated discs appear normal compared control samples. Signal for FLNC appears normal in the RV but reduced in the LV.

Signal for connexin 43, desmoplakin, and SAP97 is reduced in both RV and LV. N-cadherin is used as a marker of tissue quality and is normal in all specimens.

Table 1

Clinical characteristics of *FLNC* null variant carriers in families A-D.

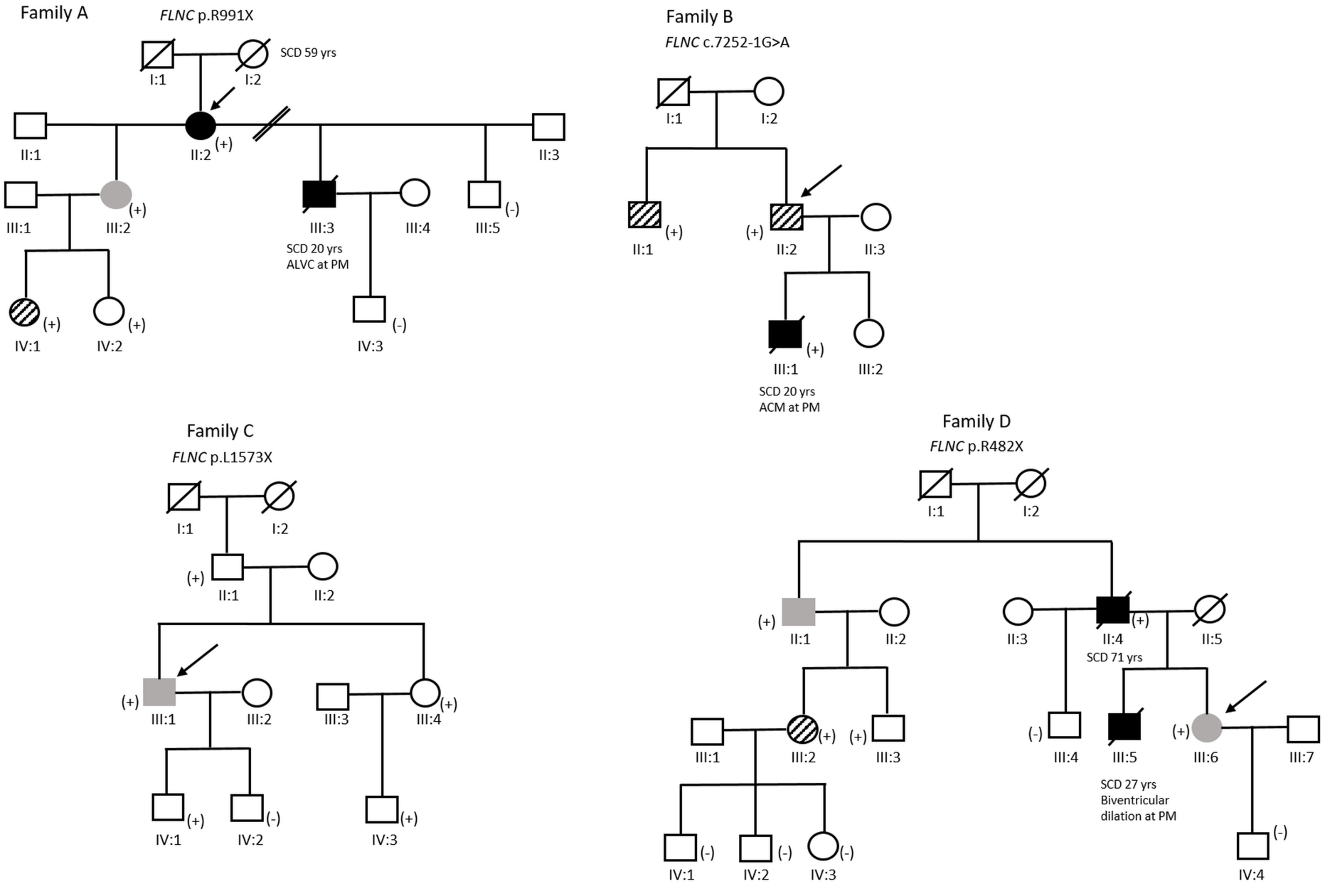
Family - Individual	*FLNC* Genotype	NYHA class / Symptoms	Age	Sex	ECG	Echocardiography		Arrhythmia	CMRI					Task Force Diagnostic Criteria (m, M) / Diagnosis
						Description	LVEF (%)	24 hr VE count / type of arrhythmia	RV EDV (ml)	RVEF (%)	LV EDV (ml)	LVEF (%)	LGE distribution	
Family A II:2 (index case)	p.Arg991X	NYHA II	76	F	Permanent AF, low QRS voltage in limb leads Late potentials	Borderline LV dilatation with mild LV systolic dysfunction	50	19,248 Non-sustained VT	146	49	122	41	Basal lateral LGE	2m and 1M Definite
Family A III:2	p.Arg991X	Asymptomatic	44	F	Low QRS voltage in limb leads	Normal biventricular size and function	55-60	12,935	Normal	Normal	172	52	Normal / No LGE	1m and 1M Borderline
Family A IV:1	p.Arg991X	Palpitations	20	F	T-wave inversion inferior leads	Normal biventricular size and function	55-60	69 Sustained VT presentation, RBBB morphology	212	54	206	59	Basal lateral sub-epicardial LGE	2m Possible
Family A IV:2	p.Arg991X	Asymptomatic	18	F	Unremarkable	Normal biventricular size and function	55-60	1	168	58	184	56	Normal / No LGE	1m Unaffected

Family B II:1	c.7252-1G>A	Presyncope	64	M	Incomplete RBBB	Normal biventricular size and function	55-60	535						2m Possible
Family B II:2 (index case)	c.7252-1G>A	Asymptomatic	62	M	Low QRS voltage	Normal biventricular size and function	55-60	22	210	61	209	65	Sub-epicardial LGE basal-mid anterolateral and inferolateral walls	1M* Possible
Family C II:1	p.L1573X	Asymptomatic	76	M	Loss of inferior R waves	Normal biventricular size and function	59	0	147	67	156	64	Basal lateral epicardial LGE	None Unaffected
Family C III:1 (index case)	p.L1573X	Syncope	54	M	Low QRS voltage in limb leads	Non-dilated LV with mild LV dysfunction RV regional wall motion abnormality (dyskinetic RVOT and RV free wall)	45-50	Sustained VT of LBBB morphology with superior axis	211	51	183	51	Extensive basal to mid sub-endocardial LGE in the anterolateral and inferolateral walls	1m and 1M Borderline
Family C III:4	p.L1573X	Palpitations	50	F	T wave inversion V6	Normal biventricular size and function	60	166	Normal	Normal	112	70	Equivocal basal LGE	1m Unaffected
Family C IV:1	p.L1573X	Asymptomatic	19	M	Unremarkable	Normal biventricular size and function	59	3	180	54	173	57	Normal / No LGE	None Unaffected

Family C IV:3	p.L1573X	Asymptomatic	26	F	Poor R-wave progression in anterior leads	Normal biventricular size and function	60	0	141	64	161	60	Normal / No LGE	None Unaffected
Family D II:1	p.Arg482X	Asymptomatic	73	M	Unremarkable	Non-dilated LV and mild LV dysfunction	45	1,093	141	41	196	48	Circumferential basal LGE and mid-inferior and inferolateral LGE	1m and 1M Borderline
Family D II:4	p.Arg482X	NYHA II	SCD 71	M	T-wave inversion V6	Dilated LV and severe LV dysfunction	35	9,249 Non-sustained VT	Normal	normal	257	52	Extensive inferior and inferolateral subepicardial basal LGE	2m and 1M Definite
Family D III:2	p.Arg482X	Asymptomatic	46	F	Unremarkable	Normal biventricular size and function	60-65	6 Non-sustained VT	131	71	149	68	Normal / No LGE	2m Possible
Family D III:3	p.Arg482X	Asymptomatic	43	M	Unremarkable	Normal biventricular size and function	60-65	3	161	57	146	68	Subtle streak of non-ischaemic LGE in the basal inferolateral wall	1m Unaffected
Family D III:6 (index case)	p.Arg482X	NYHA II	50	F	Low QRS voltage in precordial leads	Borderline LV dilatation with mild to moderate LV systolic dysfunction	40-45	5,197	227	62	187	62	Basal inferolateral and inferior wall LGE	1m and 1M Borderline

AF, atrial fibrillation; CMRI, cardiac magnetic resonance imaging; SCD, sudden cardiac death; ECG, electrocardiogram; EDV, end diastolic volume; EF, ejection fraction; NYHA, New York Heart Association classification; LBBB, left bundle branch block; LGE, late gadolinium

enhancement; LV, left ventricle; LVEF, left ventricular ejection fraction; LVIDD, end-diastolic internal dimension; m, minor Task Force ARVC diagnostic criterion; M, major Task Force ARVC diagnostic criterion; RBBB, right bundle branch block; RV, right ventricle; RVEF, right ventricular ejection fraction; RVOT, right ventricle outflow tract; VE, ventricular ectopic; VT, ventricular tachycardia; * one major diagnostic criterion due to family history.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I:2
II:1
II:2
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II:3
III:1
III:2
(+)
III:3
SCD 20 yrs
ALVC at PM
III:4
III:5
(-)
(+)
(+)
(-)
IV:1
IV:2
IV:3
Family B
FLNC c.7252-1G>A
I:1
I:2
(+)
(+)
II:1
II:2
II:3
(+)
III:1
III:2
SCD 20 yrs
ACM at PM
Family C
FLNC p.L1573X
I:1
I:2
(+)
II:1
II:2
(+)
III:1
III:2
III:3
(+)
III:4
(+)
(-)
(+)
IV:1
IV:2
IV:3
Family D
FLNC p.R482X
I:1
I:2
(+)
II:1
II:2
II:3
(+)
II:4
SCD 71 yrs
II:5
III:1
(+)
III:2
(+)
III:3
(-)
III:4
III:5
SCD 27 yrs
Biventricular
dilation at PM
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III:6
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(-)
(-)
(-)
(-)
IV:1
IV:2
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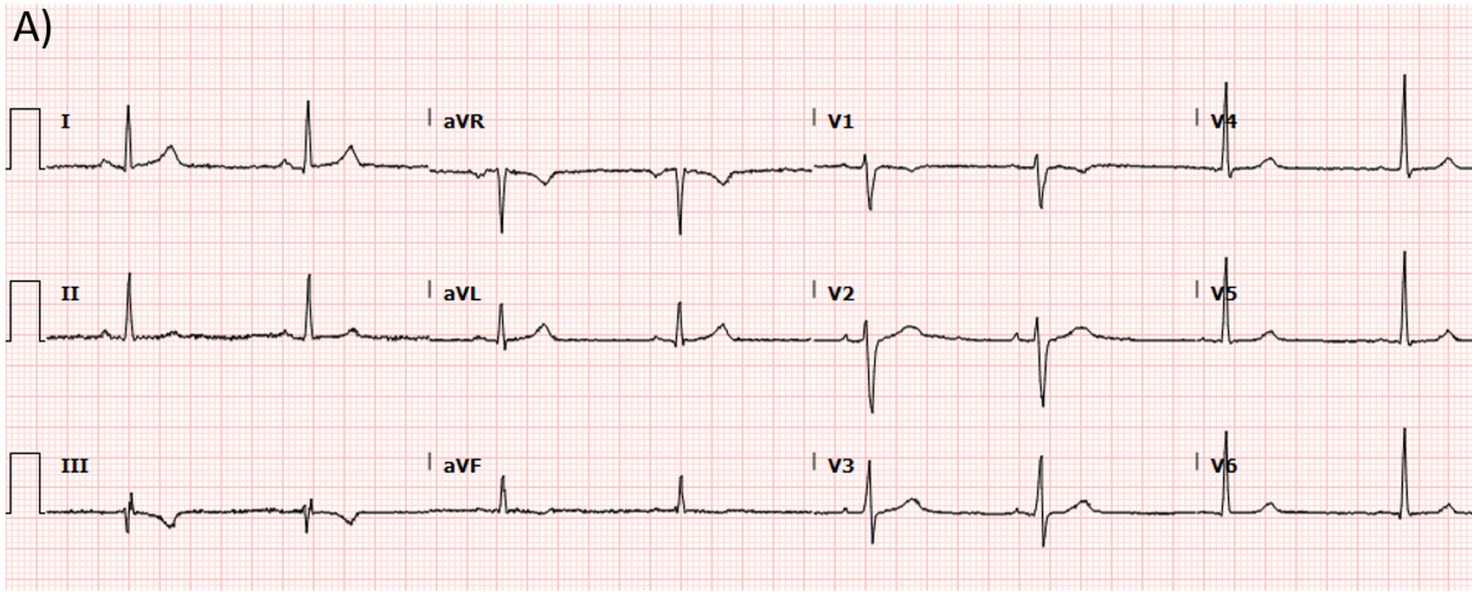
IV:4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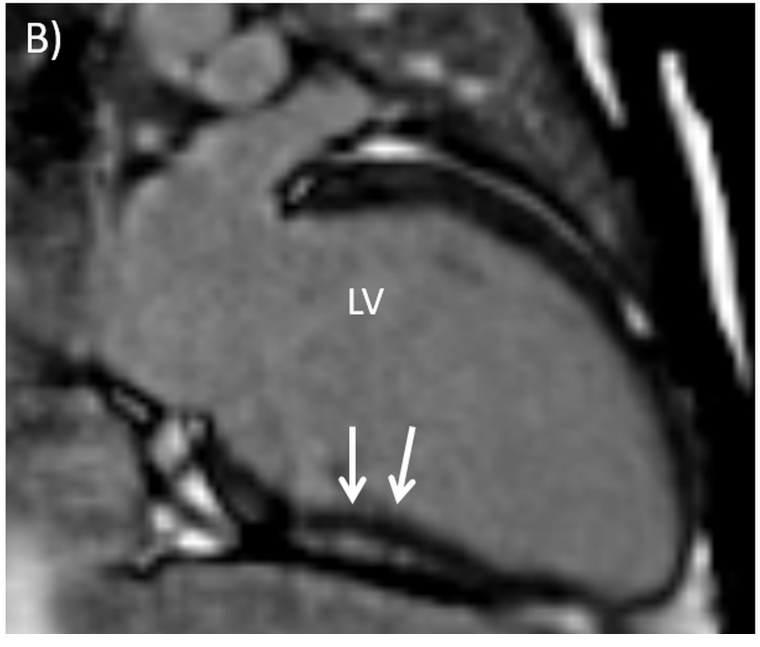
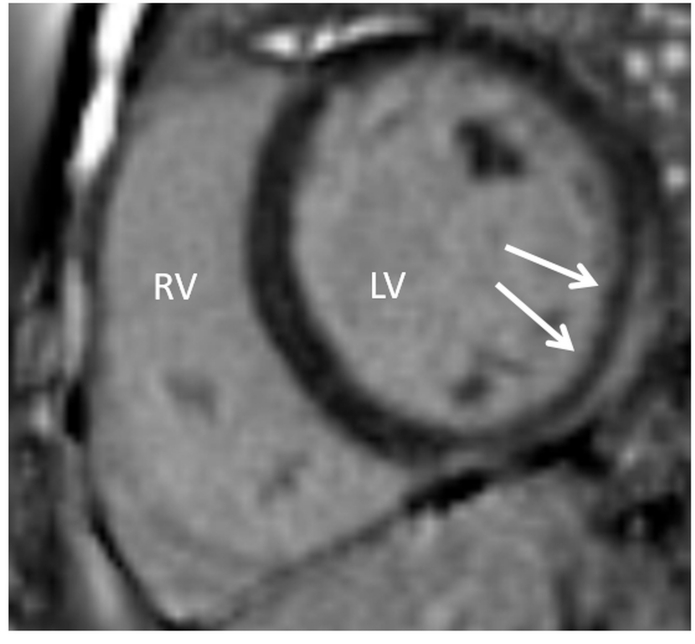
V6


B)
LV


RV
LV

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Manuscript Title: Filamin C variants are associated with a distinctive clinical and immunohistochemical arrhythmogenic cardiomyopathy phenotype

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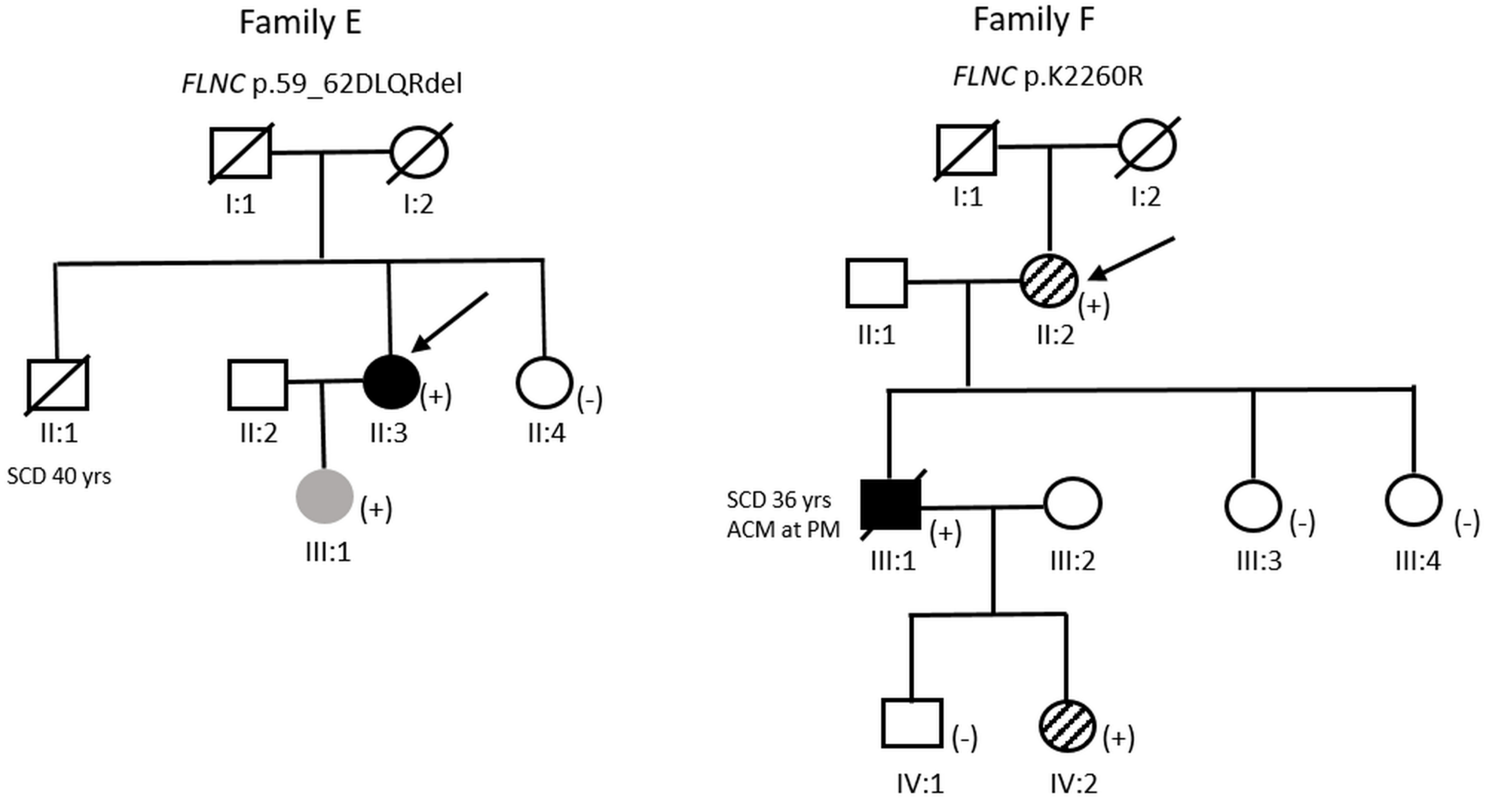
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(+)
II:4
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(+)
III:1
Family F
FLNC p.K2260R
I:1
I:2
II:1
(+)
II:2
SCD 36 yrs
ACM at PM
(+)
III:1
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III:4
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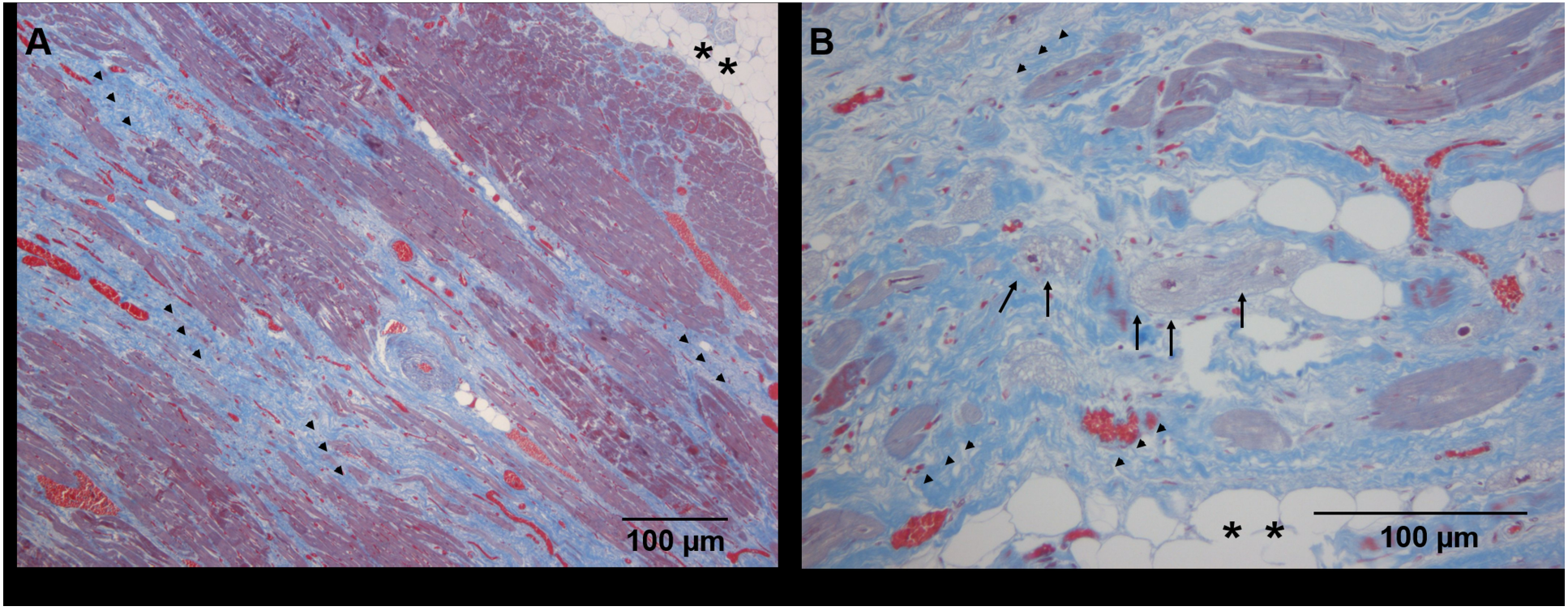
(+)
IV:2


A
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Filamin C variants are associated with a distinctive clinical and immunohistochemical arrhythmogenic cardiomyopathy phenotype

Charlotte L. Hall, Mohammed M. Akhtar, Maria Sabater-Molina, Marta Futema, Angeliki Asimaki, Alexandros Protonotarios, Chrysoula Dalageorgou, Alan M. Pittman, Mari Paz Suarez, Beatriz Aguilera, Pilar Molina, Esther Zorio, Juan Pedro Hernández, Francisco Pastor, Juan R. Gimeno, Petros Syrris, William J. McKenna

Appendix

Supplementary methods

Clinical testing

ECGs were recorded at rest using a standard protocol (10mm/mV in speed 25mm/s) in the standard lead position. Incomplete RBBB was defined in this study as QRS width <120ms with an R wave peak time in V1 or V2>50ms. Low voltage was defined as QRS voltage less than 5mm in all limb leads and less than 10mm in all precordial leads. Poor R-wave progression was defined by R wave height ≤ 3mm in V3. Epsilon waves (reproducible low-amplitude signals between the end of QRS complex and the onset of T wave) and T-wave inversion were studied on all leads. T-wave inversion was defined as T-waves of negative amplitude ≥0.1mV. QRS duration in leads V1–V6 and terminal activation duration of QRS complex (TAD) in leads V1–V3 (from the nadir of S wave to the end of QRS, including R′) were measured. Signal averaged electrocardiograms were performed using time-domain analysis with a bandpass filter of 40Hz in individuals with QRS complex duration <110ms on standard ECG. 24h Holter monitoring was recorded on an outpatient basis. Ventricular extrasystoles and episodes of ventricular tachycardia (≥3 consecutive ventricular complexes at a rate of ≥100 beats/min) were

noted. No individual was receiving antiarrhythmic or other drugs known to affect the QRS complex at the time of acquisition of the ECG tracings.

All individuals underwent echocardiography included standard 2-dimensional measurements of left ventricular end-diastolic diameter (LVEDd) and left ventricular ejection fraction (LVEF) by the Simpson bi-plane method. Henry's formula was used to correct dimensions for age and body surface area. Right ventricular (RV) outflow-tract end-diastolic diameter was measured on parasternal long-axis view (RVOT-PLAX). Wall motion abnormalities (hypokinesia, akinesia, dyskinesia, and aneurysm) of the right and left ventricles were documented.

CMRI was being performed on a 1.5 Tesla scanner (Magnetom Avanto, Siemens Medical Solutions) using a cardiac 32-channel phased array coil. Balanced steady-state free precession cine imaging are used to acquire 10–12 short axis slices (8mm slice thickness, 2mm gap) with one slice per breath-hold. Four-chamber, two-chamber and LV inflow/outflow views and a short-axis stack from mitral annulus to apex were obtained. Sequence parameters are 1.5ms echo time, 3.1ms repetition time, and acquired voxel size usually were 1.9 × 1.9mm with a typical FOV of 350mm in the phase encoding direction. Late gadolinium enhancement (LGE) imaging was acquired with a standard segmented 'fast low-angle shot' two-dimensional inversion-recovery gradient echo sequence or a respiratory motion-corrected, free-breathing single shot SSFP averaged phase sensitive inversion recovery (PSIR) sequence at 15 minutes following the injection of 0.1 mmol/kg of a gadolinium based contrast medium. Volumetry and all tissue characterisation analyses were performed on a standard post-processing platform (cvi42, version 5.6.5, Circle Cardiovascular imaging, Calgary, Canada). Manual epicardial and endocardial contours were drawn on the LV-SAX stack to measure LV volumes at end-diastole and end-systole. Papillary muscles and trabeculae were included within the LV cavity volume. LGE was deemed present if viewed on SAX stack imaging with verification in one LAX view, LGE will be recorded on 16 segments according to the American Heart Association 17 segment

model (segment 17 not assessed) along with the location (sub-endocardial, sup-epicardial, mid-wall or transmural) and pattern of fibrosis.

Bioinformatics analysis of WES data and variant filtering

Paired-end sequence reads were aligned with NovoAlign (Novocraft Technologies) against the reference human genome assembly GRCh37 (hg19). Duplicate reads removal, format conversion and indexing were performed with Picard (http://picard.sourceforge.net/). The Genome Analysis Toolkit (GATK) (https://www.broadinstitute.org/gatk/) was used to recalibrate base quality scores and to perform local realignments around possible indels. The HaplotypeCaller 3.1 package in GATK was used to call variants and to generate a multi-sample joint genotyping.

Variants were annotated using ANNOVAR software [1] and the Variant Effect Predictor (VEP) [2] tool from Ensembl. Pathogenicity of the identified missense variants was predicted using multiple bioinformatics *in silico* tools, namely HumVar-trained PolyPhen-2 model [3], SIFT [4] and MutationTaster [5].Variants were also annotated with frequencies as reported in large sequencing studies, with the Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org/) [6] being the largest. Variants identified by WES with a minor allele frequency (MAF) higher or equal to 0.001 (0.1%) in the publically available gnomAD database were removed from further analysis. Variants outside of the coding and splice site regions were also filtered out. Remaining genetic changes were filtered by their predicted functional effect, which prioritized variants that are likely to result in a loss-of-function (stop gain, frameshift deletion or insertion), non-synonymous or altering splicing.

Copy number variants (CNVs) analysis

The analysis of large rearrangements in the coding regions of genes was performed using a read depth strategy designed to overcome biases associated with sequence capture and high-

throughput sequencing. This set of tools is implemented in the ExomeDepth software package (freely available at the Comprehensive R Archive Network) [7].

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (5μm) were deparaffinised, dehydrated, rehydrated and heated in citrate buffer (10mmol/l, pH 6.0) to enhance specific immunostaining. After being cooled to room temperature, the tissue sections were simultaneously permeabilized and blocked by incubation in phosphate-buffered saline (PBS) containing 1% Triton X-100, 3% normal goat serum and 1% bovine serum albumin [8]. The sections were then incubated first with a primary antibody and then with indocarbocyanine-conjugated goat anti-mouse or anti-rabbit rabbit IgG. Primary antibodies included mouse monoclonal N-cadherin (Sigma, concentration 1:400), rabbit polyclonal Cx43 (Sigma, 1:400), mouse monoclonal plakoglobin (Sigma, 1:1000), mouse monoclonal desmoplakin (Fitzgerald, 1:10), rabbit polyclonal SAP97 (Santa Cruz Biotechnology, 1:50), rabbit polyclonal anti-GSK3β (Cell Signaling Technology, 1:80) and rabbit monoclonal anti-FLNC (Abcam, 1:200).

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Fig. A1. Pedigrees of ACM families with *FLNC* VUS variants.

Squares indicate males; circles, females; slashes, deceased individuals; black symbols, definite diagnosis of ARVC based on current Task Force diagnostic criteria [9] or evidence of ARVC at post mortem; grey symbols, borderline diagnosis of ARVC; hatched symbols, gene positive individuals with possible diagnosis of ARVC; (+), positive genotype for *FLNC* variant; (-), negative genotype for *FLNC* variant. Arrows indicate the index case in each family. PM, post mortem; SCD, sudden cardiac death. Gene negative individuals with a possible diagnosis of ARVC based solely on family history are depicted as unaffected.

Fig. A2. Masson's trichrome staining histology images of left ventricular myocardium obtained at post mortem from a carrier of the R1370X *FLNC* variant (case no 8) showing A). Fatty (asterisks) and fibrous infiltration (arrow heads) (x250) and B). Additionally patchy degeneration of cardiomyocytes (arrows) (x400).

Fig. A3. Summary of immunohistochemistry staining data for RV and LV fixed tissue specimens from ACM cases with *FLNC* variants.

Table A1

FLNC variants identified by WES in cohort of ACM patients without pathogenic variants in known cardiomyopathy and arrhythmia genes.

Sample ID	*FLNC* cDNA change	FLNC amino acid change	gnomAD frequency [6]	ACMG classification [10, 11]
Family A II:2	c.2971C>T	p.R991X	Novel	Pathogenic
Family B II:2	c.7252-1G>A	Predicted abnormal exon splicing*	Novel	Pathogenic
Family C III:1	c.4718T>A	p.L1573X	Novel	Pathogenic
Family D III:6	c.1444C>T	p.R482X	Novel	Pathogenic
Family E II:3	c.174-185del	p.59_62DLQRdel	Novel	VUS
Family F II:2	c.6779A>G	p.K2260R	4.554×10^{-5}	VUS
Index case G	c.6173A>G	p.Q2058R	Novel	VUS
Index case H	c.2141T>C	p.I714T	Novel	VUS
Index case I	c.5644A>G	p.I1882V	0.001122	VUS

Variant classification according to ACMG guidelines was performed using the InterVar online tool [10, 11]. VUS, variant of unknown significance. *predicted effect on exon splicing by NETGENE2 (http://www.cbs.dtu.dk/services/NetGene2/) [12] and Berkeley Drosophila Genome project (BDGP; http://www.fruitfly.org/seq_tools/splice.html) [13].

Table A2

Incidents of sudden cardiac death in families with *FLNC* variants.

Family	Documented SCD (n)	Age at death (yrs)	PM report	Comment
A	2	20	ALVC	Individual III:3
		59	N/A	Not shown in pedigree
B	1	20	ACM	Individual III:1 *FLNC* c.7252-1G>A carrier
C	3	40	N/A	Not shown in pedigree
		60	N/A	Not shown in pedigree
		70	N/A	Not shown in pedigree
D	3	27	Biventricular dilatation	Individual III:5
		29	N/A	Individual II:5
		71	N/A	Individual II:4 Definite diagnosis, *FLNC* R482X carrier
E	1	40	N/A	Individual II:1

F	1	36	ACM	Individual III:1 *FLNC* K2260R carrier
G	2	26	ACM	Pedigree not shown
		60	N/A	Pedigree not shown
H	1	42	N/A	Pedigree not shown
I	1	47	N/A	Pedigree not shown

ACM, arrhythmogenic cardiomyopathy; ALVC, arrhythmogenic left ventricular cardiomyopathy; PM, post mortem; SCD, sudden cardiac death.

Table A3

Clinical characteristics of *FLNC* VUS variant carriers in cases E-I.

Family - Individual	*FLNC* Genotype	NYHA class- Symptoms	Age	Sex	ECG	Echocardiography		Arrhythmia	CMRI					Task Force Criteria (m, M) / Diagnosis
						Description	LVEF (%)	24 hr VE count / Type of arrhythmia	RV EDV (ml)	RVEF (%)	LV EDV (ml)	LVEF (%)	LGE distribution	
Family E II:3 (index case)	p.59_62 DLQRdel	palpitations NYHA I	68	F	Inferolateral T-wave inversion, late potentials	Moderate to severe LV dysfunction RV regional wall abnormality (dyskinesia plus RV dilatation)	35-40	AF, Non-sustained VT	N/A	N/A	N/A	N/A	N/A	3m and 1M Definite
Family E III:1	p.59_62 DLQRdel	Asymptomatic	38	F	Unremarkable	Unremarkable	55	750, Non-sustained VT	N/A	N/A	N/A	N/A	N/A	1m and 1M Borderline
Family F II:2 (index case)	p.K2260R	Asymptomatic	74	F	Low QRS voltage and poor R wave progression, SAECG normal	Unremarkable	55	1	89	69	77	75	Sub-epicardial at basal mid to inferior wall	1M Possible
Family F IV:2	p.K2260R	Asymptomatic	16	F	Unremarkable	Unremarkable	60	0	Normal	Normal	141	63	No LGE	1M* Possible
Index case G	p.Q2058R	Palpitations	67	F	Low QRS voltages	Unremarkable	55	paroxysmal AF, 84	Normal	Normal	112	69	Basal lateral non-infarct fibrosis	1M* Possible
Index case H	p.I714T	NYHA II and Syncope	64	M	T-wave inversion V1-V4	Dilated RV, RV lateral wall akinesia	50	138	290	44%	227	57	No LGE	1m and 2M Definite

								Non-sustained VT						
Index case I	p.I1882V	NYHA I	78	M	Unremarkable ECG Late potentials	Moderate RV dilatation and RV aneurysm	55	N/A	N/A	N/A	N/A	N/A	N/A	1m and 2M* Definite

AF, atrial fibrillation; CMRI, cardiac magnetic resonance imaging; ECG, electrocardiogram; EDV, end diastolic volume; EF, ejection fraction; NYHA, New York Heart Association classification; LGE, late gadolinium enhancement; LV, left ventricle; m, minor Task Force ARVC diagnostic criterion; M, major Task Force ARVC diagnostic criterion; RV, right ventricle; SAECG, signal averaged electrocardiogram; VE, ventricular ectopics; VT, ventricular tachycardia; * one major diagnostic criterion due to family history (pedigree not shown for index case G).

Table A4

FLNC variants identified in cardiac tissue samples used in immunohistochemistry analysis.

Case no	*FLNC* genomic or cDNA change	FLNC amino acid change	gnomAD frequency [6]	ACMG classification [10, 11]
1	g.128470694_128498579del ^ (c.3_*2del)	p.0?	Novel	Pathogenic
2	c.105G>C	p.K35N	Novel	VUS
3	c.249C>G	p.Y83X	Novel	Pathogenic
4	c.479C>A	p.T160K	Novel	VUS
5	c.1965_1966delTG	p.A656PfsX8	Novel	Pathogenic
6	c.2115_2120delTGCCCA	p.Y705X	Novel	Pathogenic
7	c.4288+2T>G	Predicted abnormal exon splicing *	Novel	Pathogenic
8	c.4108C>T	p.R1370X	Novel	Pathogenic
9	c.5398G>T	p.G1800X	Novel	Pathogenic
10	c.5398G>T	p.G1800X	Novel	Pathogenic
11	c.5298+21C>T	Predicted abnormal exon splicing *	Novel	VUS

Variant classification according to ACMG guidelines was performed using the InterVar online tool [10, 11]. VUS, variant of unknown significance. *predicted effect on exon splicing by NETGENE2 (http://www.cbs.dtu.dk/services/NetGene2/) [12] and Berkeley Drosophila Genome project (BDGP; http://www.fruitfly.org/seq_tools/splice.html) [13]. ^Genomic coordinates of the *FLNC* gene according to Ensembl human genome assembly GRCh37.p13.