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CLINICAL RESEARCH
Heart failure/cardiomyopathy

The *TMEM43* Newfoundland mutation p.S358L causing ARVC-5 was imported from Europe and increases the stiffness of the cell nucleus

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Aims Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a rare genetic condition caused predominantly by mutations within desmosomal genes. The mutation leading to ARVC-5 was recently identified on the island of Newfoundland and caused by the fully penetrant missense mutation p.S358L in *TMEM43*. Although *TMEM43*-p.S358L mutation carriers were also found in the USA, Germany, and Denmark, the genetic relationship between North American and European patients and the disease mechanism of this mutation remained to be clarified.

Methods and results We screened 22 unrelated ARVC patients without mutations in desmosomal genes and identified the *TMEM43*-p.S358L mutation in a German ARVC family. We excluded *TMEM43*-p.S358L in 22 unrelated patients with dilated cardiomyopathy. The German family shares a common haplotype with those from Newfoundland, USA, and Denmark, suggesting that the mutation originated from a common founder. Examination of 40 control chromosomes revealed an estimated age of 1300–1500 years for the mutation, which proves the European origin of the Newfoundland mutation. Skin fibroblasts from a female and two male mutation carriers were analysed in cell culture using atomic force microscopy and revealed that the cell nuclei exhibit an increased stiffness compared with *TMEM43* wild-type controls.

Conclusion The German family is not affected by a *de novo TMEM43* mutation. It is therefore expected that an unknown number of European families may be affected by the *TMEM43*-p.S358L founder mutation. Due to its deleterious clinical phenotype, this mutation should be checked in any case of ARVC-related genotyping. It appears that the increased stiffness of the cell nucleus might be related to the massive loss of cardiomyocytes, which is typically found in ventricles of ARVC hearts.

Keywords Arrhythmogenic right ventricular cardiomyopathy • Sudden cardiac death • TMEM43 • Molecular genetics • Cardiogenetics

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Introduction

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is associated with myocardial fibrosis, fibrofatty replacement, and a progressive loss of predominantly right ventricular tissue, although biventricular disease involvement is not uncommon. Ventricular arrhythmias may cause sudden cardiac death (SCD) in ARVC patients. Disease causing mutations in genes encoding for desmosomal proteins have been reported in more than 40–50% of ARVC patients.[1,2]

A rare form of ARVC is caused by a missense mutation within the gene of transmembrane protein 43 (*TMEM43*) on chromosome 3p25 (ARVC-5). In a total of 15 Canadian families, a heterozygous amino acid substitution (p.S358L) in *TMEM43* fully cosegregated with autosomal dominant ARVC and was proposed to be a founder mutation on the island of Newfoundland (Canada).[3] *TMEM43*-p.S358L is a fully penetrant mutation. Although the mutation is not located on the heterosomes, the phenotype is strikingly gender specific.[4] Males have a lower life expectancy[3,4] than female carriers and carry a high risk for SCD. The frequency of ARVC-5 in central Europe is currently unknown. Recently, a first European family with this mutation was independently identified in Denmark[5] and almost at the same time in Germany.[6] However, the Danish family did not have male carriers of the mutation.[5] A *de novo* mutation was also recently found in a Canadian cohort with obviously non-Newfoundland individuals.[7]

The gene *TMEM43* codes for the nuclear protein LUMA, which appears to form a complex with the nuclear lamina proteins lamin A/C and emerin,[8] which respective genes are known to underlie Emery Dreifuss Muscular Dystrophy (EDMD). Mutations in *LMNA* coding for lamin A/C were also identified in patients with arrhythmogenic forms of dilated cardiomyopathy (DCM) and ARVC.[9] LUMA is a ubiquitously expressed nuclear protein and was predicted to carry four transmembrane domains which localize the protein to the inner nuclear membrane. Consequently, other *TMEM43* variants were recently found in patients with EDMD.[10]

In this study, we describe the screening of the entire gene *TMEM43* for ARVC-associated mutations within previously genetically characterized unrelated 22 ARVC index patients[1] and analysis of the *TMEM43*-p.S358L mutation in additional 22 DCM patients. We describe the clinical course and history of a German family who was tested positive for the *TMEM43* c.1073C>T, p.S358L mutation (*Table 1*). Furthermore, we provide evidence that the Newfoundland *TMEM43*-p.S358L mutation was imported by immigrants from continental Europe. Finally, we found that in skin fibroblasts of three p.S358L mutation carriers, the nuclear nanomechanics is substantially affected.

Methods

Patient cohort

We studied 22 unrelated ARVC index patients already characterized for the lack of mutations in the genes *DSC2*, *DSG2*, *PKP2*, *JUP*, *DSP*, and *DES*.[1] About 80% were inpatients from the Heart and Diabetes Center North Rhine Westfalia, Bad Oeynhausen, Germany (HDZ-NRW; www.hdz-nrw.de), whereas the remaining patients were ambulatory patients referred to for molecular genetic analysis to our centre. The clinical diagnosis was classified according to the revised task force criteria for ARVC.[11] All coding exonic and adjacent intronic sequences of the *TMEM43* gene were screened.

In addition, we screened 22 patients with DCM recruited from the Heart- & Diabetes Center NRW, Bad Oeynhausen, Germany, for the presence of c.1073C>T, p.S358L in *TMEM43* using a sequence-specific TaqMan SNP genotyping assay. Dilated cardiomyopathy patients were included in the study, since recently in 5% of DCM patients desomosmal gene variants associated with ARVC were also found[12] indicating that the phenotype in cardiomyopathies is not strictly associated with a particular gene.[9]

Genetic analysis

Genomic DNA was isolated from peripheral blood samples according to the standard protocols. DNA of blood samples was extracted using standard techniques (Illustra^{TM} blood genomic Prep Mini Spin Kit, GE Healthcare, Bucking Hamshire, UK). The genomic sequence used to design polymerase chain reaction (PCR) primers was obtained from GenBank (www.ncbi.nih.gov/projects/genome/guide/human; accession-No.: NM_024334). Polymerase chain reaction amplification of genomic DNA was carried out following standard protocols. Mutation screening was done by denaturing high-performance liquid chromatography using a DNASep column with a WAVE DNA Fragment Analysis System (Transgenomic Inc., San Jose, CA, USA) as described previously.[13] The analytical temperature(s) and primers for each exon are available from the authors upon request. Exons with aberrant temperature-modulated heteroduplex profile were sequenced in both directions on an ABI 310 genetic analyzer using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing electropherograms were inspected manually and analysed with the software *Variant Reporter* (Applied Biosystems).

To evaluate a potential founder effect, 16 microsatellite markers spanning *TMEM43* on chromosome 3p25 were selected for haplotype analysis. Primers and conditions are available upon request. In addition to the family described here, haplotype analysis was also performed in three additional families from Denmark, three index-patients from the original Newfoundland ARVC-5 founder family, and a patient from the USA. The different families were not known to be related. In patients DK1, DK2, DK3 and GER1 DNA of relatives of the index patients, we facilitated the verification of the phase and reconstruction of haplotypes.

To estimate the age of the mutation, the linkage disequilibrium between the p.S358L mutation and each of the closest recombinant microsatellite markers was calculated and the recombination fraction from the distances between the mutation and these microsatellite markers was determined. This enabled the calculation of the number of generations since the mutation had occurred.[14] The genetic distances (cM) were inferred from the deCODE genetic map.[15]

Cell cultures of dermal fibroblasts

Dermal fibroblasts from individual IV/5 (*Figure 1*), from 2 Danish male *TMEM43*-p.S358L carriers, and from *TMEM43* wild-type controls were isolated from skin biopsy as previously reported.[16] Skin biopsies of control individuals were isolated from two unrelated healthy volunteers, three subjects with inherited DCM due to the mutations *LMNA* p.K219T, *PKP2* p.H679Y, and *PLN* p.R14del, and two patients with the non-cardiac diseases osteogenesis imperfecta and chronic granulomatosis (for clinical data see Supplementary material online, *Table S1*). All control cell cultures were wild type for the *TMEM43* mutation.

The culture medium was DMEM (Life Technologies, Darmstadt, Germany) supplemented with 10% foetal bovine serum (PAA laboratories, Cölbe, Germany) 4 mmol/L glutamine, 4.5 g/L glucose, 50 μM 2-mercaptoethanol (Life Technologies, Darmstadt, Germany), 1% (w/v)

Table 1 **Clinical classification [21] of *TMEM43*-p.S358L mutation carriers**

Clinical parameters	TF criterion	Pat. III/01	Pat. III/03	Pat. IV/01	Pat. IV/03	Pat. IV/05	Pat. IV/6	Pat. IV/8	Pat. IV/10	Pat. V/01	Pat. V/02	Pat. V/03
Gender		F	F	M	M	F	M	F	F	F	M	M
Age (years)		74	68	37*	32*	46	28*	35	33	15	12	9
Family history												
Autopsy		−	−	+	+	−	+	−	−	−	−	−
SCD		−	−	+	+	−	+	−	−	−	−	−
Pathogenic mutation (*TMEM43*-p.S358L)	Major	±	±	±	±	±	±	±	±	±	±	±
Depolarization abnormalities												
TAD ≥55 ms	Minor	−	n.a.	−	−	−	n.a.	n.a.	n.a.	−	−	−
Epsilon waves	Major	−	n.a.	−	−	−	n.a.	n.a.	n.a.	−	−	−
Late potentials	Minor	−	n.a.	−	−	−	n.a.	n.a.	n.a.	−	−	−
Repolarization abnormalities												
Inverted T in V1-3	Major	−	n.a.	−	−	−	n.a.	n.a.	n.a.	−	−	−
Inverted T in V4-6	Minor	−	n.a.	−	−	−	n.a.	n.a.	n.a.	−	−	−
Arrhythmias												
VES >500/24 h	Minor	+	+ (ICD)	n.a.	+	+ (ICD)	n.a.	+ (ICD)	n.a.	−	−	−
LBBB VT	Minor	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	−	−
LBBB VT sup.	Major	−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	−	−
RBBB VT		−	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	−	−	−
Structural characteristics												
Structural major	Major	+	n.a.	+	+	−	+	+	n.a.	−	−	−
RV WMA		+	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	−	−	−
RV DE		+	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	−	−	−
LV WMA		−	n.a.	n.a.	n.a.	+	n.a.	n.a.	n.a.	−	−	−
LV DE		−	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	−	−	−
Functional characteristics												
RVEF ≤ 45%		−	n.a.	n.a.	n.a.	−	n.a.	+	n.a.	−	−	−
LVEF ≤ 50%		−	n.a.	n.a.	n.a.	−	n.a.	n.a.	n.a.	−	−	−
Ventricular involvement												
RV involvement		+	n.a.	+	+	−	+	n.a.	n.a.	−	−	−
LV involvement		+	n.a.	+	+	−	−	n.a.	n.a.	−	−	−

Continued

Table 1 *Continued*

Clinical parameters	TF criterion	Pat. III/01	Pat. III/03	Pat. IV/01	Pat. IV/03	Pat. IV/05	Pat. IV/6	Pat. IV/8	Pat. IV/10	Pat. V/01	Pat. V/02	Pat. V/03
No. of major TF criteria		2	1	2	2	1	2	2	1	1	1	1
No. minor TF criteria		1	1	0	1	1	0	1	0	0	0	0
Concluding ARVC diagnosis[21]		Definite	$Border-line	Definite	Definite	$Border-line	$Definite	$Definite	$Possible	Possible	Possible	Possible

F, female; ICD, implanted cardioverter defibrillator; LBBB VT, ventricular tachycardia with left bundle branch block morphology; LV DE, LV delayed enhancement; LV WMA, left ventricular wall motion abnormalities (a/dyskinaesia); M, male; n.a., not available; RBBB VT, ventricular tachycardia with right bundle branch block morphology; Structural major, structural RV abnormalities accounting for a major criterion; RV WMA, right ventricular wall motion abnormalities (a/dyskinaesia); RV DE, RV delayed enhancement; SCD, sudden cardiac death (<35 years); sup. , superior axis; TAD, terminal activation duration; TF, Task force; VES, ventricular extra systoles; *, at death; $, classification biased by incomplete clinical data; −, absence or +, presence of clinical parameter; ±, heterozygous genotype. Tissue characterization was not available, since cardiac tissue was not preserved during autopsy for histomorphometric analyses.

on-essential amino acids (Life Technologies), 1% (w/v) penicillin (100 U/mL) and streptomycin (100 μg/ml), and 25 mM HEPES.

Analysis of nuclear nanomechanics

Local force indentation curves of skin fibroblasts were acquired with a MFP3D (Asylum Research, Goleta, CA, USA) atomic force microscope (AFM) which was mounted on an inverted optical microscope (Olympus IX 71, ×40 phase contrast objective) and allowed precise alignment of the cantilever tip relative to the probed cell nucleus of interest (*Figure 3A*) and to observe cell motility (for details of the methods, see Supplementary material online).

We observed a nice agreement of predicted and experimental data in the analysis region which validates the applicability of the Hertz elasticity model (Supplementary material online, *Figure S5*). All indentation curves were recorded and analysed in a blinded fashion with respect to the genotype of the cell cultures.

Results

Genetic analysis

We identified in our cohort the mutation *TMEM43*-p.S358L as published before in Newfoundland[3] in a single German family with 20 members including 3 male SCD victims (*Figure 1*). The family decided after genetic counselling to test all available members of the family. Predictive testing was done in accordance with the recommendations of the ESC working group and the German law.[17] The heterozygous under-aged patients V/01-03 without cardiac phenotype are being followed using ECG, Holter ECG, echocardiography including tissue Doppler and speckle tracking as well as magnetic resonance imaging on a regular basis. Until now, no abnormalities have been detected in children and adolescents below 18 years in this family. Disease classification is based in these individuals on the presence of the mutation only (*Table 1*). DNA sequencing of the coding region including the exon–intron boundaries of the genes *DSP*, *PKP2*, *DES*, *DSG2*, *DSC2*, and *JUP* revealed the heterozygous variant *DSP* c.8531G>T, p.G2844V in patients III/01, III/03, IV/05, IV/08, and IV/09, but not in IV/11 and especially not in the SCD victims IV/01 and IV/06.

Thus, this variant did not cosegregate with disease in this family and was therefore regarded to be not disease causing. The index patient III/01 was additionally screened for variants in the candidate genes *PLN*,[18] *LMNA*,[9] *CTNNA3*,[19] *CDH2*, and *CTNNB1*,[20] but no relevant pathologic sequence variants related to the familial cardiomyopathy were found (Supplementary material online, *Table S2*). The genetic screening for the known desmosmal ARVC mutations and further candidate genes remained therefore negative.

Arrhythmogenic right ventricular cardiomyopathy was confirmed in all three male SCD victims at autopsy. Two of those died during physical exercise. The female family members IV/05, IV/08, and IV/09 presented with ventricular tachyarrhythmias leading to ICD implantations in IV/05 and IV/08. The family reported that II/02 had frequent episodes of arrhythmias and died from SCD aged 78 years. The grandfather (I/01) of the index patient (III/01) died as well from SCD (see arrow in *Figure 1*). In addition, patient III/03 received an ICD as a consequence of a near fatal syncope. The *TMEM43* mutation cosegregated with the disease in the family and was confirmed in paraffin embedded tissue samples of two male

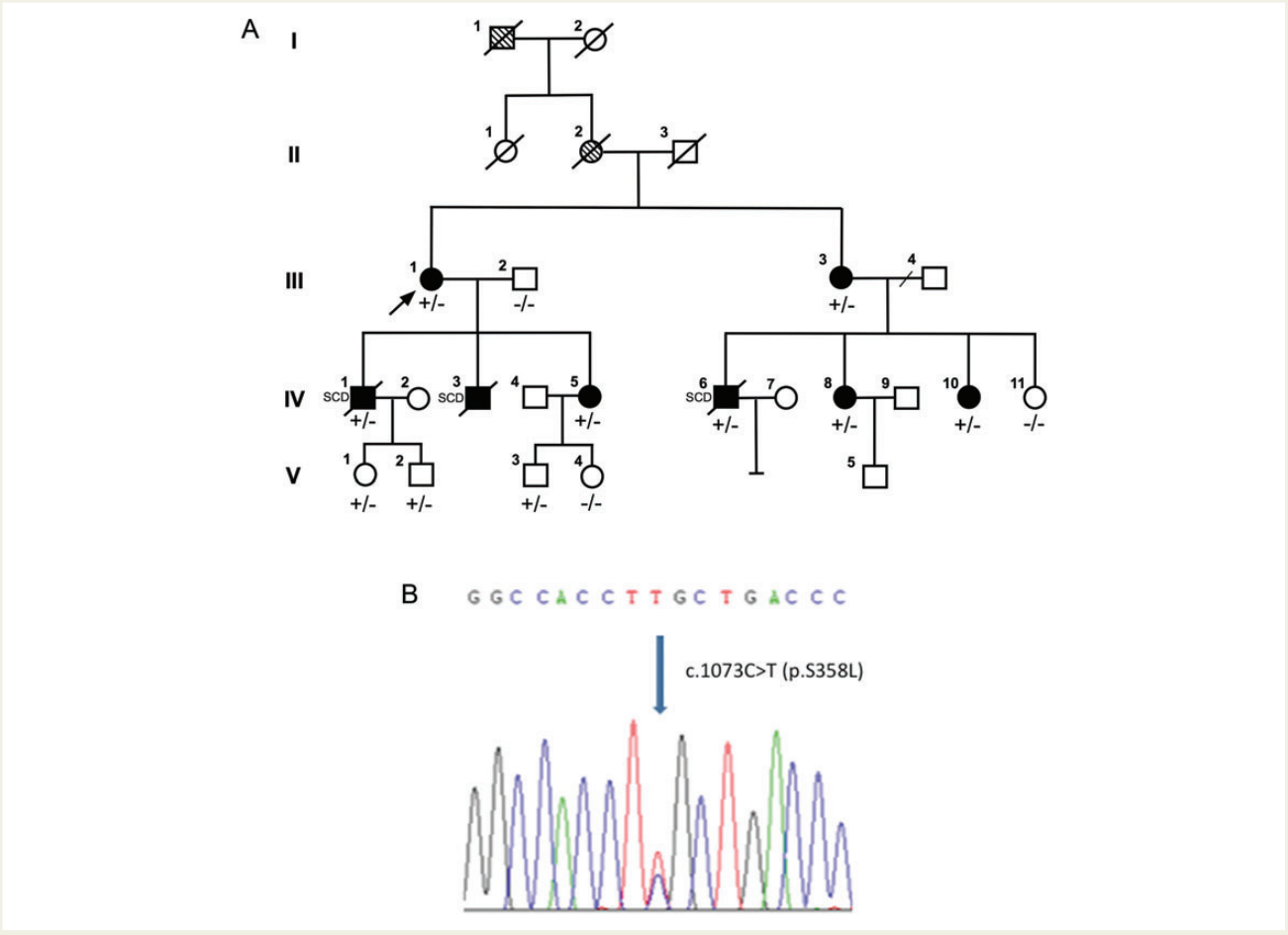

Figure 1 (*A*) Family tree of the German family with the mutation *TMEM43*-p.S358L. All male patients of generation IV died due to sudden cardiac death (SCD) despite being under medical control without knowledge of the genetic background. Hatched symbols mark individuals suspected to be diseased. Filled symbols represent the cardiac phenotype. The heterozygous genotype *TMEM43*-pS.358L is given as (+/−). (*B*) Sanger sequencing chromatogram of index patient III/01 showing the heterozygous mutation *TMEM43* c.1073C>T, p.S358L.

relatives (IV/01 and IV/06), who died by SCD (aged 37 and 32 years, respectively). No samples for genotyping were available from autopsy for individual IV/03. However, a transthoracic echocardiogram performed 9 months before death of IV/03 revealed a mildly enlarged right ventricle in the absence of detectable aneurysms (right ventricular end-diastolic diameter, RVEDD = 33 mm,) while LV dimensions were normal (left ventricular end-diastolic diameter, LVEDD = 55 mm). The patient revealed during examination frequent ventricular ectopic beats. During autopsy, fibrofatty infiltrations in the right ventricular free wall were detected. His brother (IV/01) died suddenly without antecedent cardiac symptoms, while his sister (IV/5) had frequent episodes of tachyarrhythmias and received finally an ICD. However, she presented with a right ventricle with a dyskinaesia of the posterior wall and a mild systolic dysfunction during MRI examination. In general, the female mutation carriers of the family had no signs of heart failure and expressed a less severe phenotype than male carriers. ECGs of the family members III/01, IV/01, IV/03, and IV/05 were available as Supplementary material online. We found that the QRS times of individuals IV/03 and IV/05 were in the upper normal range (~100 ms), whereas the QT interval was normal in all individuals tested (compare also *Table 1* for clinical ARVC-classification[21] of the mutation carriers).

Of note, the phenotype of the family perfectly recapitulated the gender-specific severity of the disease with an increased risk for SCD in male mutation carriers as reported before.[3,4] Interestingly, mutation carriers of the German *TMEM43* family revealed in addition to the cardiac disease a phenotype of idiopathic lipodystrophy. None of the patients in the German family had a dermatological phenotype. The allele frequency of the p.S358L mutation in our patient cohort was calculated to be 1.7%. In addition, we did not find the mutation in any DCM patient. Due to the lack of cosegregation, the DSP variant was not considered as pathogenic. We did also not find any evidence for a digenic inheritance or modifier effect of this variant.

Haplotype analysis

We compared the haplotypes of the patients III/01 and IV/05 (*Figure 1*; family GER1) to the index patients from Denmark (DK1-3), USA, and Canada (Newfoundland; NFL1-3). The haplotype analysis in eight different *TMEM43*-p.S358L mutation carriers revealed that all index patients shared a 1 Mb region around *TMEM43*, with larger

A

Marker	Position (Mb)	DK1		DK2		DK3		GER1		USA1		NFL1		NFL2		NFL3	
D3S1259	12.10	197	205	205	203	203	201	191	185	191	205	203	203	203	203	203	195
D3S3610	13.01	248	258	258	248	248	244	244	248	244	258	248	248	248	248	244	258
D3S2403	13.17	252	258	258	252	252	252	252	258	252	258	252	280	252	280	252	252
D3S1516	13.65	331	365	365	331	361	365	331	353	331	351	347	357	347	357	347	347
D3S3608	13.70	251	249	251	251	249	249	249	249	249	249	249	249	249	249	249	249
D3S2385	13.88	149	145	149	139	149	145	149	149	145	145	149	145	149	145	149	145
D3S3602	13.92	114	120	114	114	114	120	114	120	114	120	114	120	114	120	114	120
D3S1585	13.94	121	123	121	119	121	127	121	129	121	127	121	127	121	129	121	129
TMEM	p.S358L	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
D3S1554	14.36	131	131	131	135	131	131	131	135	131	135	131	131	131	131	131	125
D3S3595	14.64	266	268	266	268	266	268	266	268	266	270	266	266	266	266	266	266
D3S3613	15.36	199	203	199	189	199	189	189	181	191	185	195	199	195	203	195	205
D3S3473	16.52	223	221	223	221	223	217	217	221	217	219	217	221	217	217	217	221
D3S2338	16.84	176	182	176	186	176	186	186	182	186	184	186	186	186	182	186	182
D3S4547	17.95	235	219	235	247	235	235	235	231	239	227	235	219	235	219	235	235
D3S3510	19.09	283	281	283	277	283	281	281	277	283	281	283	281	283	281	283	281
D3S1293	21.92	131	117	131	117	131	117	117	117	117	125	125	125	125	125	125	127

B

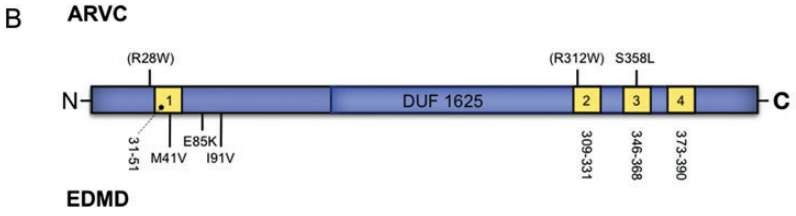

Figure 2 (*A*) Shared haplotype surrounding the gene *TMEM43* in p.S358L mutation carrying patients. The table shows constructed shared haplotypes among different families. In families DK1, DK2, DK3, and GER1 (indicated with *), additional affected family members were available to reconstruct the phase of the haplotype. In gray are depicted the shared markers between different families. In light blue are marked specific markers present in the Danish population, in yellow specific markers in the German and US patients, and in green the markers specific for the Newfoundland population. In patient DK2, a cross-over may have occurred. (*B*) Schematic map of known *TMEM43* variants associated with Emery Dreifuss Muscular Dystrophy (EDMD) or arrhythmogenic right ventricular cardiomyopathy (ARVC). Variants with unknown significance[33] were given in brackets. The domain structure was derived from ref. 8.

shared haplotypes among specific populations, e.g. in patients from Denmark/Germany or in the different Newfoundland index-patients. These data suggest a shared common haplotype indicative for an ancient founder mutation (*Figure 2*). Interestingly, a member of the Danish family (DK1) left Denmark for Canada in the early nineteenth century.

Age of the mutation

A shared haplotype for four markers in a 0.7 Mb (1.3 cM) region surrounding *TMEM43* was found in all p.S358L mutation carriers. The linkage disequilibrium between the p.S358L mutation and recombinant markers D3S2385 and D3S3613 was calculated. This revealed that the p.S358L mutation occurred between 52 and 64 generations ago. Allowing 25 years per generation, the age of the haplotype containing the p.S358L mutation is therefore estimated to be between 1300 and 1600 years (corresponding to the years 400–700 AD).

Cell nucleus nanomechanics of dermal fibroblasts

LUMA was found to be a nuclear envelope protein[8,22] (see also Supplementary material online, *Figures S3* and *S4*), which appears to be expressed ubiquitously. For that reason, we investigated fibroblasts from a skin biopsy of patient IV/05 and two Danish male TMEM43-p.S358L carriers using AFM. We analysed the expression of the mRNA and the protein coded by *TMEM43* by reverse transcription polymerase chain reaction (RT-PCR), immunoblotting and -staining, respectively (Supplementary material online, *Figures S2* and *S3*). To compare the relative expression of LUMA in dermal fibroblasts and the myocardium, we analysed by real-time RT-PCR the mRNA expression levels (Supplementary material online, *Figure S1A* and *B*). In addition, we stained the fibroblast cell cultures by two different commercially available antibodies against LUMA (Supplementary material online, *Figure S3*). In summary, there is evidence that LUMA is expressed in human skin fibroblasts on a level comparable with that of the human myocardium.

We isolated dermal fibroblasts and characterized the nuclear nanomechanical resistance and stiffness by AFM force indentation experiments in cell culture. We compared the cell culture data from three heterozygous *TMEM43*-p.S358L carriers to fibroblasts of healthy controls or different non-cardiac diseases. As a further control, we also tested fibroblasts from patients with DCM (DCM due to *LMNA*-p.K219T, due to *PLN*-p.R14del, and related to a novel variant *PKP2*-p.H679Y). All control cells were tested to be wild type for *TMEM43*.

In order to determine the stiffness of the cell nuclei, we acquired more than 12 000 force indentation curves (*Figure 3B* and *C*) for each patient (30 different cells with 400 indentation curves each) and calculated the elastic moduli thereof. These elastic data for each cell were plotted in a histogram (*Figure 3D*). We found that

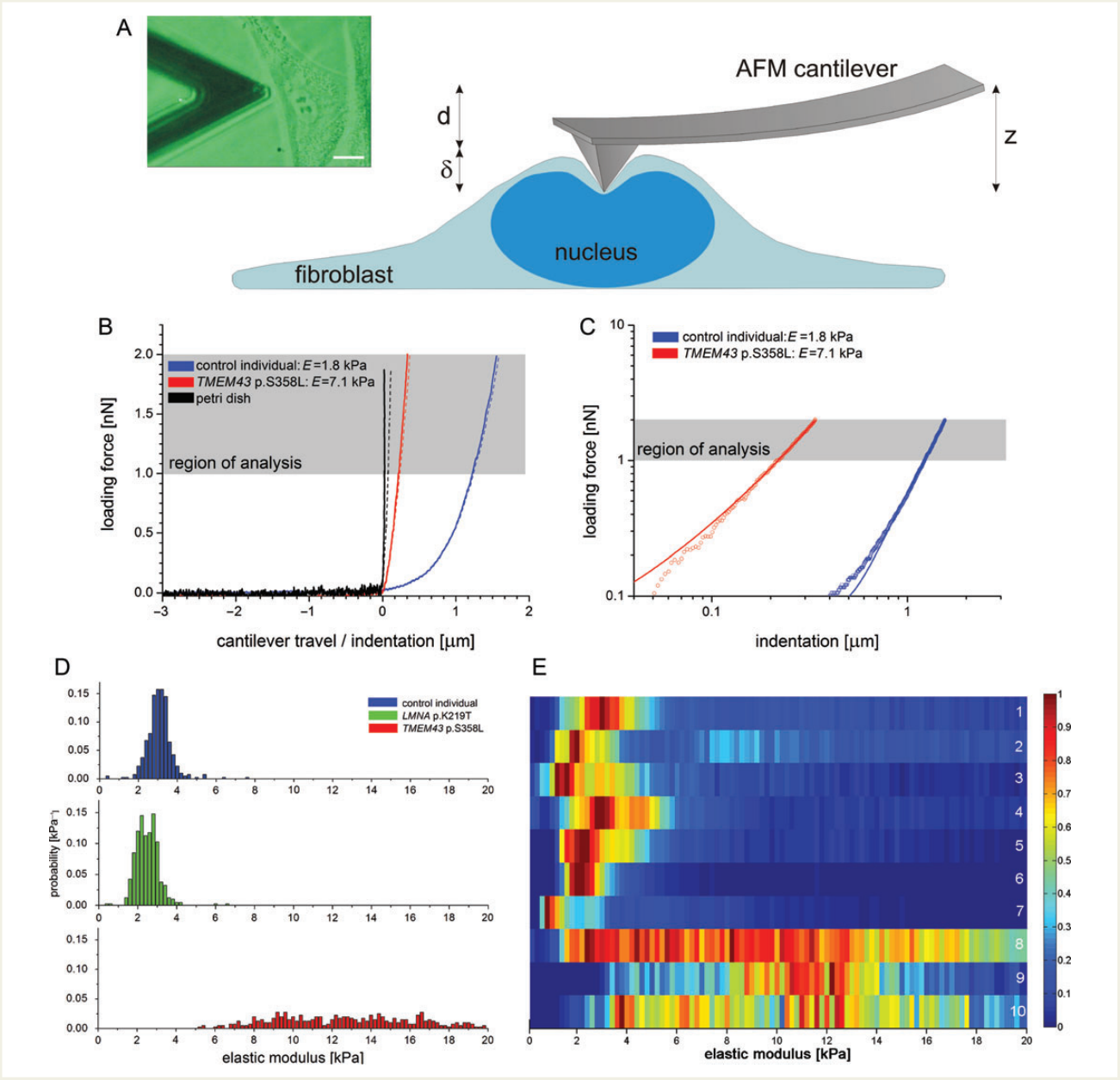

Figure 3 Measurement of nuclear stiffness by atomic force microscopy (AFM). (*A*) Schematic view of a cell indentation experiment using AFM. The cell is indented by the AFM cantilever and is deflected due to the elastic mechanic response of the cell. The cantilever movement *z* is equal to the sum of indentation δ and deflection *d* ($z = \delta + d$), whereas on an inflexible surface (not shown here) the cantilever deflection *d* corresponds to the cantilever movement *z* ($z = d$). Inset: Light microscopy of a single skin fibroblast and a triangular AFM cantilever. (*B*) Force distance curves ($F(z)$, dashed) acquired on substrates with different stiffness: control cell nucleus (blue), *TMEM43* p.S358L nucleus (red), and bottom of cell culture petri dish (black). The cantilever is approached (from left) and touches the sample surface at zero. Further approaching of the cantilever leads to both a deflection of the cantilever and an indentation of the sample. The sample indentation can be estimated by subtracting the deflection from the cantilever travel ($F(z) \rightarrow F(\delta)$). The resulting force indentation curves ($F(\delta)$, solid lines) allow the direct read out of the sample deformation. (*C*) Experimental cell indentation data (open circles) in a log–log plot is approximated by Sneddon's equation (solid lines) to estimate the elastic modulus *E*. (*D*) Representative elasticity histograms derived from an indentation scan of a single cell. Each histogram is normalized to an overall probability of 1. The elastic properties of the skin fibroblast of *TMEM43*-p.S358L patient IV/5 (red) differs remarkably from those of a healthy control (blue) or from dilated cardiomyopathy due to the mutation *LMNA*-p.K219T (green). (*E*) Colour-coded histograms of the elastic modulus from different patients. Each distribution is a cumulative of at least 30 cell nuclei containing 400 indentation curves each. The maximum of every histogram is set to 1 for better visibility. Non-cardiomyopathy controls: (**1**) healthy control A, (**2**) healthy control B, (**3**) chronic granulomatosis, and (**4**) osteogenesis imperfecta. Skin fibroblasts from cardiomyopathy patients: (**5**) DCM (mutation *PLN*-p.R14del), (**6**) DCM (mutation *LMNA*-p.K219T) (**7**) DCM (homozygous mutation PKP2- p.H679Y), (**8**) ARVC-5 (mutation *TMEM43*-p.S358L, female), and (**9–10**) ARVC-5 (mutation *TMEM43*-p.S358L, males).

the elasticity data from single control cells showed a characteristic Gaussian shape distribution with a peak value of 1–3 kPa (elastic modulus) and a comparably small standard deviation of $\sigma < 1$ kPa (*Figure 3D*).

In full contrast, the nuclear stiffness and elasticity of cell nuclei derived from patients with *TMEM43*-p.S358L differed significantly from those of the controls (*Figure 3A–E*). Our data evidence that the nanomechanical properties of the nuclei in *TMEM43*-p.S358L cells exhibit a considerably higher average stiffness with an increased elastic modulus ($E \approx 10$ kPa). Moreover, the scattering of the stiffness data in *TMEM43*-p.S358L cells is much broader compared with those of the wild-type controls. This difference of the nanomechanical properties was observed throughout the whole analysis independent of the gender of the mutation carrier (*Figure 3E*): All wild-type control nuclei were significantly softer, exhibiting a considerably narrower distribution of the elastic moduli (*Figure 3D* and *E*).

As a consequence, the mutation *TMEM43*-p.S358L heavily affects the nanomechanical properties of the cell nucleus. Thus, nuclei bearing the mutant LUMA protein are substantially stiffer than nuclei from the control group. Of note, in very rare cases (<5%), significantly stiffer cell nuclei were also observed in control cells (data not shown). However, the shape and the standard deviation of these distributions remained unchanged and are attributed to mechanical phenomena related to cell division and proliferation.[23]

Furthermore, and as an additional control, we imaged the orientation of the nucleosomes before and after an indentation scan. Therefore, we could guarantee not to observe any signs for nucleosomic displacement during the experiments (see Supplementary material online, *Figure S6*).

Discussion

Arrhythmogenic right ventricular cardiomyopathy is a rare cardiomyopathy[24] affecting preferentially the right or both ventricles and is regarded to be a genetic trait in the majority of cases. Variants in genes coding for desmosomal genes are frequently found to be associated with this cardiomyopathy.[24–27] However, of most variants the molecular disease mechanism is not yet known[27] and the pathogenic role of some missense variants is still a matter of debate.[28,29] Moreover, variants in genes coding for proteins of the cardiac desmosome are also found in patients with different forms of cardiomyopathy like i.e. DCM.[12,30] It is to be expected that novel genes will be identified in ARVC patients, since the hit rate in genetic screening studies of the known disease-causing genes is ~40–50%.[1,2]

Recently, the gene responsible for ARVC-5[31] was identified as *TMEM43*.[3] This gene does not code for a desmosomal protein but the inner nuclear envelope protein LUMA, which was initially identified by a proteomics analysis of nuclear lamina proteins.[22] The missense mutation *TMEM43*-p.S358L was shown in a cohort from the island Newfoundland to be a fully penetrant mutation leading to ARVC, heart failure, and SCD in affected individuals.[3] They also found evidence that this mutation is a founder mutation.

Settlement of Newfoundland started in the middle of the eighteenth century. By 2001, ~98% of the population in Newfoundland was of English or Irish descent.[32] Thus, it was suggested that the mutation was imported from the British islands at the beginning of the 18th century.[4] However, Haywood *et al.* failed to identify mutation carriers with *TMEM43*-p.S358L analysed in a British ARVC cohort,[4] although they found the novel variants p.R28W, p.E142K, and p.R312W, which are, however, of unknown significance [variant of unknown significance (VUS); Fig. *2B*]. Interestingly, they found also some haplotypes, which were in common with the Newfoundland patients.[4]

We recently identified the novel mutation p.N116S in the gene *DES*, which caused ARVC in a young female patient with terminal heart failure.[1] In that cohort, we had a hit rate of ~40% for variants related to the disease in the genes *DSG2*, *DSC2*, *DES*, *JUP*, *PKP2* and *DSP2*. As a consequence of the work of Merner *et al.*,[3] we decided to screen this cohort again for variants in *TMEM43* and identified the Newfoundland mutation *TMEM43*-p.S358L in a German family.[6] Strikingly, the phenotype of the disease was similar to the disease course of the Newfoundland families: the males died by SCD aged between 28 and 37 years, while females were affected by arrhythmias (*Figure 1A*). Another European family was published almost at the same time by Christensen *et al.*[5] in Denmark. Thus, apparently unrelated families carrying the Newfoundland mutation were identified in different countries of continental Europe.

A relationship with the Newfoundland families could not be assessed at the first glance. According to reports from the family, it was assumed that the grandfather I/01 of the index patient (*Figure 1A*), who died from SCD, was the first mutation carrier within this family. However, the data of our haplotype analysis revealed that the three European families and mutation carriers from Newfoundland and the USA have a common genetic haplotype, suggesting that the German mutation is not a spontaneous *de novo* mutation but was inherited from a common European ancestor. Therefore, it is likely that additional mutation carriers will be identified on the European continent.

This conclusion is supported by the finding that the mutation appears to be an old continental variant dating back to the early medieval age (years 400–700 AD) and long before the today's European nations were built. Surprisingly, the estimated frequency of this mutation in Europe is comparably low (*TMEM43*-p.S358L in the general population ranging from 1:460 000 to 1:1 250 000 or 1:230 among European ARVC cases), when other European *TMEM43*-screening studies[5,6,33] or data on the global prevalence of ARVC[24] were considered. The comparably high incidence of ARVC-5 on the island Newfoundland might be related to the genetic drift of this unfavourable variant in larger populations. However, at present we can only speculate why the prevalence of *TMEM43* p.S358L on the island of Newfoundland appears to be considerably higher compared with the European continent.

TMEM43 encodes the protein LUMA, which is a transmembrane protein of the inner membrane of the nuclear envelope. In a series of experiments, Bengtsson and Otto[8] provided evidence that LUMA is in a molecular complex with the nuclear envelope proteins lamin and emerin. Both proteins carry mutations in patients with EDMD. Thus, LUMA is part of the 'linkers of nucleoskeleton and cytoskeleton complex' (LINC), which connects the nuclear lamina with the cytoskeleton.[34] Consequently, other mutations in *TMEM43* were also recently found in patients with EDMD,[10] who are frequently affected by mutations in *LMNA* and *EMD*. However, the EDMD related *TMEM43* mutations p.E85K and p.I91V were found in the N-terminal portion of the protein, which resides in the

lumen of the endoplasmic reticulum (*Figure 2*). When these mutants were transfected in HeLa cells, the localization of lamin B was not affected but a considerable number of cells had an irregular shape of the nucleus.[10] The VUS recently identified in the British ARVC cohort[33] was also found at the N-terminus (p.R28W), located in the nucleoplasma, or within the transmembrane domain 2 of LUMA (p.R312W) (*Figure 2*). Of note, the Newfoundland mutation p.S358L is located within the third transmembrane domain of LUMA.

Recently, Rajkumar *et al.*[35] demonstrated that transfection of cells with the cDNA of *TMEM43* did not affect the expression of lamin B and emerin transcripts or structural properties of the cell nucleus. Thus, currently the precise pathomechanism of the Newfoundland mutation is unknown.

Mutations in genes coding for nuclear envelope proteins and especially variants in *LMNA* are associated with EDMD, DCM, pathologies of the nervous system and adipose tissue, or the Hutchinson Gilford progeria syndrome (for a review see ref. 36). Of note, lamin, encoded by *LMNA,* is a ubiquitously expressed protein and its myopathy related mutations were analysed in fibroblast model systems previously.[37,38] Since LUMA was found to be a binding partner of lamin,[8] we speculated that the mutation *TMEM43*-p.S358L might also affect the nuclear nanomechanics of cells. Therefore, we recorded the nanomechanical properties of the cell nuclei in skin derived fibroblast cultures derived from patient IV/5 and two Danish male mutation carriers. We found that in contrast to different *LMNA*[38] mutations, the nuclei of the *TMEM43*-p.S358L cells show stiffer nanomechanical characteristics compared with controls derived from skin fibroblasts of two voluntary control individuals, two patients with non-cardiac disease (osteogenesis imperfecta and chronic granulomatosis), and three patients with DCM due to (i) the homozygous mutation *PKP2*-p.H679Y, (ii) *PLN*-p.R14del, and (iii) inherited DCM due to the mutation *LMNA*-p.K219T. We found that the data of the nanomechanical analysis could be fitted in the control cells by a Gaussian distribution, but not in the mutant *TMEM43*-p.S358L cells revealing that the mutant LUMA leads to aberrant and increased mechanical stiffness of the nuclei. In contrast to these findings, Zwerger *et al.*[38] recently published a comparative analysis of *LMNA* mutants, which are associated with DCM, EDMD, partial lipodystrophy, and Charcot–Marie–Tooths disease. They found that skin fibroblasts derived from myopathic patients with the *LMNA* mutations p.ΔK32 and p.E358K were associated with aberrant nuclear stability leading to softer cell nuclei. However, some of the myopathic mutants like i.e. *LMNA*-p.E203G in the recombinant model were not associated with changed nuclear mechanics,[38] which is in line with our observation on the *LMNA* mutant p.K219T (*Figure 3E*).

Our observation is also in agreement with previous findings in recombinant LUMA-transfected cells. Liang *et al.*[10] observed an abnormal nuclear shape in HeLa cells, when transfected with *TMEM43* cDNAs coding for the EDMD mutations p.E85K and p.I91V. Thus, in contrast to myopathic *LMNA* mutations,[38] *TMEM43*-p.S358L leads to increased stiffness of the cell nucleus, which might lead to a stochastic death of cardiomyocytes. It appears that the increased stiffness as well as the elevated elasticity of the cell nucleus might not be tolerated in mechanically active cells like cardiomyocytes.

We did not find nanomechanical differences *in vitro* between cell cultures of males or females. This might be explained by a systemic effect *in vivo*, hormonal effects, or the time scale of the cell culture experiments in comparison with the chronic effects in mutation carriers. Of note, the increased incidence of SCDs among male mutation carriers parallels the natural course of the testosterone plasma concentrations in young men. In this hormonal context, a mechanistic gender effect was also suggested by a recent paper of Arimura *et al.*[39] who identified a testosterone effect in recombinant mice carrying the homozygous *Lmna* mutation p.H222P, which codes for the aberrant version of the nuclear protein lamin. It is currently not known whether hormonal effects are also responsible for the gender phenotype of *TMEM43*-p.S358L carriers. Gender effects related to exercise were also found by a recent study of James *et al.*[40] They clearly demonstrated the clinical effects of endurance and frequent exercise in desmosomal gene mutation carriers. This potentially might also explain gender differences in the clinical phenotype of *TMEM43*-p.S358L carriers. However, the deceased male mutation carriers of the German family did only recreational sports for about 2–4 h per week on average. Thus, the exercise effects do not provide valid evidences for the clinical gender differences in this family. Thus, the reasons for the clinical gender differences in ARVC-5 remain currently unclear.

In summary, we conclude and predict from our data that

(1) The mutation *TMEM43*-p.S358L is more widely distributed worldwide than anticipated before and therefore it is expected that further mutation carriers will be identified in Europe. Therefore, due to full penetrance and malignancy, all ARVC patients should be tested for the presence of this mutation.
(2) The history of the German family reveals that without the knowledge of the mutation, the individual risk for SCD in ARVC patients might be clinically underestimated.
(3) The mutation affects the stability of the nucleus due to increased stiffness, which might be associated with cellular death.

Supplementary material

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

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