Prevalence and significance of rare RYR2 variants in arrhythmogenic right ventricular cardiomyopathy/dysplasia: results of a systematic screening.


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Article Type: Original-clinical-Genetic

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Abstract: ABSTRACT

Background
Arrhythmogenic right ventricular Cardiomyopathy/Dysplasia (ARVC/D) is a genetic disease predominantly caused by desmosomal gene mutations that account for only ~50% of cases. RYR2 gene mutations usually cause Catecholaminergic Polymorphic Ventricular Tachycardia (CPVT) but have been associated with peculiar phenotype named ARVC2.

Objectives
We aim to determine the prevalence and phenotype associated with RYR2 mutations in a large ARVC/D population.

Methods
We analyzed the whole RYR2 coding sequence by Sanger sequencing in 64 ARVC/D probands without desmosomal gene mutations.

Results
We have identified six rare missense variants p.P1583S, p.A2213S, p.G2367R, p.Y2932H, p.V3219M and p.L4670V. It corresponds to a prevalence of 9% of rare RYR2 variants in ARVC/D population (6 probands/64) that is significantly higher than the estimated rate of rare RYR2 variants in control (Fisher test, p=0.03). Phenotypes associated with RYR2 variants were similar to desmosome-related ARVC/D, associating typical ECG abnormalities at rest, frequent monomorphic ventricular tachycardia, right ventricular dilatation, wall motion abnormalities and fibro-fatty replacement when histopathological examination was available.

Conclusion
In this first systematic screening of the whole coding region of the RYR2 gene in a large ARVC/D cohort without mutation in desmosomal genes, we show that putative RYR2 mutations are frequent (9% of
ARVC/D probands) and are associated with a conventional phenotype of ARVC/D, in contrast with previous findings. The results support the role of RYR2 gene in conventional ARVC/D.
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Manuscript Number or Title: SIGNIFICANCE OF RYR2 MUTATIONS IN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPAHY/DYSPLASIA: A SYSTEMATIC SCREENING

Signed:/Date 2013/11/15
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Print Name: Roux-Buisson
Dear Editor,

Please find enclosed our revised manuscript entitled "PREVALENCE AND SIGNIFICANCE OF RARE RYR2 VARIANTS IN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY/DYSPLASIA: RESULTS OF A SYSTEMATIC SCREENING " by Roux-Buisson et al., which we submit for publication in Heart Rhythm as an original article.

I would like to thank the reviewers for their constructive comments. I added a “clinical perspectives” paragraph as requested. You will find below a detailed answer to the reviewer’s comments and also the revised manuscript. I hope the reviewers and yourself will find this new manuscript to your satisfaction.

Thank you in advance for the consideration given to our work.

Yours sincerely,

Philippe Charron, MD, PhD, Assoc. Prof.
Reviewer #1:

We thank the reviewer for his/her comments.

- The stated P value of 0.003 by Fisher is important and should also appear in the abstract.

**Answer:** We modified the manuscript as recommended by the reviewer:
Abstract, page 4, lines 17-18
“It corresponds to a prevalence of 9% of rare RYR2 variants in ARVC/D population (6 probands/64) that is significantly higher than the estimated rate of rare RYR2 variants in control (Fisher test, p=0.03).”

- In the abstract, the comment: "not overlapping with CPVT" is an overstatement and should be removed.

**Answer:** We modified the manuscript as recommended by the reviewer:
Abstract, page 5, line 5
“In this first systematic screening of the whole coding region of the RYR2 gene in a large ARVC/D cohort without mutation in desmosomal genes, we show that putative RYR2 mutations are frequent (9% of ARVC/D probands) and are associated with a conventional phenotype of ARVC/D, in contrast with previous findings.”

- The limitations section should include the fact that the authors limited their analysis to the 1994 Task Force criteria, and indicate why.

**Answer:** The reviewer is right. We modified the manuscript as suggested:
Abstract, page 18, lines 13-14
“At last, the ARVC/D probands were selected according to the 1994 Task Force Criteria as this cohort was prospectively recruited before the publication of the revised TFC.”
Reviewer #2:

We thank the reviewer for his/her comments.

The questions raised were adequately addressed.

- Probably slightly modify the title: Prevalence ...: results of a systematic screening

  We modified the title as follow: “PREVALENCE AND SIGNIFICANCE OF RARE RYR2 VARIANTS IN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY/DYSPLASIA: RESULTS OF A SYSTEMATIC SCREENING”
Title: PREVALENCE AND SIGNIFICANCE OF RARE RYR2 VARIANTS IN ARRHYTHMOGENIC RIGHT VENTRICULAR CARDIOMYOPATHY/DYSPLASIA: RESULTS OF A SYSTEMATIC SCREENING

Short title: RYR2 mutations in Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia

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The number of authors is justified by the fact that this manuscript results from a multicenter study, and there is one author from each center.

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Conflict of interest: none

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Word count: 5033
ABSTRACT

Background

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We have identified six rare missense variants p.P1583S, p.A2213S, p.G2367R, p.Y2932H, p.V3219M and p.L4670V. It corresponds to a prevalence of 9% of rare RYR2 variants in ARVC/D population (6 probands/64) that is significantly higher than the estimated rate of rare RYR2 variants in control (Fisher test, p=0.03). Phenotypes associated with RYR2 variants were similar to desmosome-related ARVC/D, associating typical ECG abnormalities at rest, frequent monomorphic ventricular tachycardia, right ventricular dilatation, wall motion abnormalities and fibro-fatty replacement when histopathological examination was available.
Conclusion

In this first systematic screening of the whole coding region of the RYR2 gene in a large ARVC/D cohort without mutation in desmosomal genes, we show that putative RYR2 mutations are frequent (9% of ARVC/D probands) and are associated with a conventional phenotype of ARVC/D, in contrast with previous findings. The results support the role of RYR2 gene in conventional ARVC/D.

Key words: Arrhythmogenic right ventricular dysplasia cardiomyopathy; RYR2 gene; mutation

Abbreviations:
AA: amino acid
ARVC/D: Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia
CPP: ethical committee (comité de protection des personnes)
CPVT: Catecholaminergic Polymorphic Ventricular Tachycardia
DNA: Deoxyribonucleic acid
ECG: electrocardiogram
EP: electrophysiologic study
EVS: Exome Variant Server database
LBBB: left bundle branch block
LV: left ventricle
MRI: Magnetic Resonance Imaging
NSVT: non-sustained ventricular tachycardia
RBBB: right bundle branch block
RV: right ventricle

RVOT: right ventricle outflow tract

RYR2: Ryanodine receptor type 2

SNP: single nucleotide polymorphism

SVT: sustained ventricular tachycardia

TFC: Task Force Criteria

TWI: T-wave inversion

Vec: ventricular ectopies

VT: ventricular tachycardia

WMA: wall motion abnormalities
INTRODUCTION

Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia (ARVC/D) is a rare cardiac muscle disorder characterised by progressive fibro-fatty replacement of the myocardium. The right ventricle is predominantly affected but left ventricular involvement is also present in more than half of the cases\(^1\). These structural alterations can lead to ventricular arrhythmias and heart failure. ARVC/D is a frequent cause of sudden death in young people and athletes. The diagnosis of ARVC/D is currently based on the presence of major and minor standardised Task Force criteria (TFC) that consider ventricular arrhythmias episodes, electrocardiographic abnormalities, right ventricular function and morphology, histopathology, family history and genetic status\(^2\).

ARVC/D is usually inherited as an autosomal dominant disease with reduced penetrance and variable expression. So far, the major genes involved in ARVC/D encode components of the cardiac desmosome: plakophilin-2 (PKP2)\(^3\), desmoglein-2 (DSG2)\(^4,5\), plakoglobin (JUP)\(^6\), desmoplakin (DSP)\(^7\) and desmocollin-2 (DSC2)\(^8,9\). Comprehensive mutation screening of the five main desmosomal ARVC/D genes can detect genetic abnormalities in at least 40-50% of probands\(^10\). Non-desmosomal genes have been also associated with ARVC/D phenotypes including the cardiac ryanodine receptor type 2 gene (RYR2)\(^11\), the transforming growth factor beta 3 gene (TGFB3)\(^12\), the TMEM43 gene\(^13\) and more recently the lamin A/C (LMNA)\(^14\), the titin (TTN)\(^15\), the desmin (DES)\(^16\) and the phospholamban (PLN)\(^17\) genes. Mutations in the RYR2 gene are usually associated with catecholaminergic polymorphic ventricular tachycardia (CPVT), a rare and severe inherited arrhythmia without structural cardiac abnormality\(^18,19\) (http://www.fsm.it/cardmoc/).

RYR2 is one of the largest human genes (105 exons) encoding a mRNA of 16365 bp (NM_021991.2). Nava et al reported in 1988 a family with autosomal dominant form of right ventricular cardiomyopathy (supported by histological data) associated with polymorphic
ventricular tachycardia induced by exercise stress testing and juvenile sudden death\textsuperscript{20}. Rampazzo et al mapped then the locus to chromosome 1q42-q43 and identified \textit{RYR2} mutations in four independent families with the same clinical presentation of ARVC/D (named ARVC/D2)\textsuperscript{11,21}. This clinical presentation differs from desmosome-related forms of ARVC/D and is rather close to CPVT because of the presence of effort-induced ventricular arrhythmias, its high penetrance and a 1:1 sex ratio. The association between typical form of ARVC/D and \textit{RYR2} mutations remains unclear and the prevalence of \textit{RYR2} mutations in the ARVC/D population remains unknown since very few mutations have been associated to ARVC/D\textsuperscript{11,22-24}.

In this study, we aimed to determine the prevalence of \textit{RYR2} mutations in a large cohort of 64 well clinically characterised ARVC/D probands for whom mutations in \textit{PKP2}, \textit{DSG2}, \textit{DSP}, \textit{DSC2} and \textit{JUP} were previously excluded. Sequencing of the entire coding region of \textit{RYR2} in these ARVC/D probands led to the identification of 6 putative missense mutations in 6 unrelated probands. The pathogenic role of the variations is discussed as well as the consequences for clinical practice.

\textbf{METHODS}

\textbf{Patients}

This multicenter prospective study included a cohort of unrelated probands recruited in France and Switzerland with a diagnosis of ARVC/D according to the TFC used at time of enrolment\textsuperscript{25} and then focussed on 64 probands for whom no mutation were identified in \textit{PKP2}, \textit{DSG2}, \textit{DSP}, \textit{JUP} and \textit{DSC2} genes\textsuperscript{10}.

Clinical evaluation of all probands was performed as described previously\textsuperscript{10} and included: evaluation of personal and familial history, physical examination, 12-lead standard ECG, standard echocardiography, cardiac Magnetic Resonance Imaging (MRI) or right ventricular (RV) angiography, 24-hours ambulatory electrocardiogram (ECG) and signal-averaged-
ECG. Baseline exercise test was performed in all probands, except in very particular situations requiring urgent therapeutic management (such as implantable cardiac defibrillator or anti-arrhythmic drugs). Electrophysiologic (EP) study was performed when considered clinically relevant by the physician, according to the following protocol: 2 different sites, 2 rates, with up to 3 extra stimuli, at baseline and with infusion of isoproterenol. In the case of cardiac transplant, pathological analysis of the explanted heart was performed. Clinical evaluation of relatives was performed when available. This study was approved by the Pitié-Salpêtrière Hospital ethical committee (CPP) and written informed consent was obtained from all individuals.

**Genetic analysis**

For each proband, the 105 exons and intron-exon junctions of the *RYR2* gene were amplified from genomic DNA (OMIM 180902, transcript: NM_021991.2, protein: 4967 AA, Q92736-1, primer sequences available upon request). The analysis of the entire coding sequence of *RYR2* was performed by direct sequencing on an ABI 3130 DNA sequencer (PE Applied Biosystems®, Foster City, USA).

When unreported variant were detected, they were searched among 400 chromosomes from ethnically matched and healthy control subjects (Caucasian n=400 or Maghrebian n=134) by direct sequencing or by denaturing high-performance liquid chromatography (Wave Transgenomic Inc®, Cambridge, USA).

Upon identification, the likelihood of a pathogenic effect of an unreported *RYR2* variant was based on (i) the absence of the variant in the control population and an allele frequency inferior to 1 / 10 000 in the databases NCBI SNPs, 1000 Genome and Exome Variant Server (http://www.ncbi.nlm.nih.gov/snp, http://browser.1000genomes.org/index.html, http://evs.gs.washington.edu/EVS), (ii) the conservation of the mutated residue among species and *RYR* isoforms and (iii) the predicted effect of the mutation by three appropriate software
(Polyphen2, SIFT, SNPsandGO http://genetics.bwh.harvard.edu/pph2/.
http://sift.bii.astar.edu.sg/, http://snps-and-go.biocomp.unibio.it/snps-and-go/). The segregation analysis within the family was performed when the relatives DNAs and clinical data were available.

Statistical Analysis

The frequency of the “genetic background noise” of rare RYR2 missense variants in the general population was estimated using data from the Exome Variant Server database (EVS). By using the coverage data available in EVS website, we find that 25938 positions covering the RYR2 exons and introns boundaries had sufficient sequence coverage to be genotyped in 5818 individuals in average (99.9% of the coding sequence). We considered as a rare missense variant, each missense variant observed at maximum 5 times among the cohort of individuals genotyped in EVS.

The occurrence of these rare variations were added together and divided by the mean of allele population to reach a theoretical prevalence of rare variations in RYR2 in EVS, used as control population. The prevalence of rare RYR2 variations in our cohort (128 alleles or 64 patients) was then compared with the prevalence of RYR2 variations found in the population analysed in EVS (11636 alleles or 5818 individuals). We decided to pool both European and African ancestry for the comparison, first since our population of patients comprises both European and African ancestry and second because frequency of rare variants of RYR2 is similar in European population and in African population. P value was calculated with Fisher’s exact test. Calculations were carried out using the graphpad software: http://www.graphpad.com/quickcalcs/contingency1/.

RESULTS

Clinical data
All probands fulfilled TFC used at time of enrolment\textsuperscript{25} and four fulfilled recent update TFC\textsuperscript{2}.

Clinical data are summarised in the tables 1 and S1. Pedigrees are presented in figure 1.

**Family A.**

The proband was a man presenting with aborted sudden cardiac death during intense effort, with ventricular fibrillation. Resting ECG showed in V1 an atypical conduction defect (figure 2) and signal-averaged ECG detected late potentials. Cardiac imaging showed RV dilatation and multifocal wall-motion-abnormalities (WMA) with mild left ventricle (LV) abnormalities (table 1). He was found to carry a heterozygous \textit{RYR2} p.P1583S variant. The familial screening detected the variation in the affected father and a sister presenting with borderline phenotype while the three healthy relatives did not carry the variation. The father displayed frequent bimorphic ventricular ectopies and significant RV abnormalities on cardiac imaging. He also developed persistent atrial fibrillation needing cardioversion. The 17 year-old sister displayed a parietal block in V1 (figure 2) and mild RV abnormalities. Exercise test, EP study or isoproterenol test did not induced significant arrhythmia in the proband and his father.

**Family B.**

The proband was an asymptomatic 43-year-old man. He fulfilled diagnosis criteria for ARVC/D with the presence of T-wave inversion (TWI) from V1 to V4, non-sustained monomorphic ventricular tachycardia (NSVT) recording during the 24-hour 3D-ECG monitoring, frequent ventricular ectopies with left bundle branch block (LBBB) morphology and superior axis and late potentials (figure 2 and 3). He displayed a mild RV dilatation with global RV hypokinesia on cardiac-MRI. Electrophysiological mapping of the right ventricle showed a limited zone of scar within the inferior wall of the RV and the EP study triggered a monomorphic sustained ventricular tachycardia (SVT) with LBBB morphology and superior axis, suggesting ventricular tachycardia (VT) originating from this scar (figure 3). The molecular analysis of \textit{RYR2} found a p.L4670V heterozygous variation, also found in his
affected sister and his asymptomatic mother (figure 2). His sister displayed TWI from V1 to V3 without evidence of morphological abnormalities or ventricular arrhythmias (figure 2, table 1 and Supplementary table 1) while their mother only displayed positive late-potentials.

**Family C.**

This 59 years old male proband was a sporadic case that presented with presyncope and spontaneous ventricular arrhythmia, corresponding to monomorphic NSVT with a LBBB morphology and inferior axis. ECG showed complete right bundle branch block (RBBB). Echocardiography and cardiac MRI showed apical and RVOT dyskinesia although the RV was not significantly enlarged. He did not reach the 2010 diagnosis criteria and was then considered with a borderline diagnosis of ARVC/D. The EP study and the isoproterenol test were negative under beta-blockers. The *RYR2* p.G2367R heterozygous variation was identified. No DNA from relatives was available.

**Family D.**

The proband was a sporadic case that presented with palpitations and several episodes of syncope since the age of 35. ECG showed TWI from V1 to V3 and late potentials (figure 2). He displayed spontaneous monomorphic sustained ventricular tachycardia of LBBB morphology. Cardiac imaging showed RV dilatation and dysfunction associated with apical and infero-basal WMA. Monomorphic NSVT with LBBB morphology and superior axis was triggered at EP study but not at exercise test and during isoproterenol test (figure 3). The p.A2213S heterozygous variation was identified in *RYR2*. One of the unaffected daughters, 12 years of age, carried the variation.

**Family E.**

Proband was a sporadic case that presented with presyncope at the age of 41. Resting ECG showed TWI from V1 to V3. The 24h-ECG monitoring detected frequent monomorphic ventricular ectopies (Vec) (figure2, table S1) decreasing during exercising. Cardiac imaging
showed WMA with dyskinesia and RV aneurysm but no RV dilatation and therefore did not reach the 2010 diagnostic criteria. The heterozygous p.V3219M variation in RYR2 was identified.

**Family F.**

Proband, a 39 years old woman, presented with palpitations and dyspnea. ECG showed epsilon wave, first-degree atrio-ventricular block, incomplete RBBB. She developed atrial fibrillation (figure 2). A 24h ECG monitoring and exercise test detected frequent polymorphic ventricular ectopies, increasing with effort (figure 2, table S1). The EP study induced a syncopal polymorphic SVT. She developed a major RV dilatation with severe tricuspid regurgitation leading to end-stage heart failure that required heart transplantation. The anatomopathological study of the explanted heart confirmed typical histological features of ARVC/D with an extensive fibro-fatty replacement of the entire RV anterior wall associated with lymphocytes infiltration and important areas of fibro-fatty replacement within the LV wall (figure 4). A heterozygous p.Y2932H variation in RYR2 was identified. Familial screening detected the variation in two asymptomatic siblings.

None of the six probands presented with bidirectional VT. Mutation carriers did not show NSVT nor SVT during exercise test (performed in three probands and two relatives) or during isoproterenol test (performed in four probands and one relative).

**Genetic analysis**


RYR2 is reported to be a relatively polymorphic gene by Jabbari et al\(^26\). We decided therefore to analyse whether the prevalence of RYR2 variant in our cohort was higher or not than in the
general population. We estimated the prevalence of the genetic background noise of \textit{RYR2} variants in the general population using the data from the Exome Variant Server. We considered as a rare \textit{RYR2} variant a variant observed at maximum 5 times in EVS, which is a conservative approach. We observed that a rare \textit{RYR2} variant was identified in 215/5818 subjects in EVS (supplementary table 2). It corresponded to a rate of rare \textit{RYR2} variants of 4% (215/5818) in EVS which was significantly lower to the 9% (6/64) observed in our cohort (Fisher test, p=0.0322).


The p.P1583S variation is located in the cytoplasmic part of the protein, outside the hot spot of mutations of \textit{RYR2}. The proline residue at position 1583 is highly conserved among species and isoforms of \textit{RYR} (figure 5). The variation was predicted to be deleterious by all prediction software (table 2), and was absent among 400 control chromosomes and from the EVS and 1000 genome databases.

The p.G2367R was located in the cytoplasmic part of the protein in the second hot spot domain of \textit{RYR2} (central domain), close to the region of interaction with FKBP12.6, a major regulatory protein of \textit{RYR2}. Moreover, this variant has been previously reported in association with hypertrophic cardiomyopathy and unexplained sudden cardiac death and functional studies showed a gain of function\textsuperscript{27,28}. This variation was not found in 400 control chromosomes.

The p.L4670V variation mapped to the transmembrane domain of the protein that participates to the pore of the calcium channel. This domain is a hot spot for \textit{RYR2} mutations, with more than 30 mutations published, all associated so far with CPVT or sudden death. Moreover, a p.L4670H variation has been recently reported in association with CPVT\textsuperscript{26}. The variation affected a highly conserved leucine residue (figure 5), was predicted as deleterious by all
prediction softwares (table 2) and was found neither in 400 control chromosomes, nor in the variant databases.

The p.A2213S, p.Y2932H, p.V3219M variations were located outside the hot spot of mutations of RYR2, and modified a not conserved amino acid. They were absent from 400 control chromosomes but the p.V3219M variant was reported with a very low frequency (<0.0001%) from Exome Variant Server database.

DISCUSSION

Pathogenicity of RYR2 variants in ARVC/D

We performed the first systematic screening of the whole RYR2 gene (105 exons and intron-exon junctions) in a well clinically characterized ARVC/D cohort, after the exclusion of affected subjects harboring pathological mutations in either of the five desmosomal genes (PKP2, DSG2, DSP, JUP, DSC2). Among the 64 ARVC/D probands, we found a prevalence of rare RYR2 variants of 9%. Based on a conservative approach including the estimation of a “genetic background noise” of 4% in RYR2, we found a significantly higher frequency of RYR2 variants in our ARVC/D cohort.

In the absence of functional studies, it remains difficult to classify any missense mutation as benign or pathogenic. Therefore, interpretation of our results was performed with considerable caution. We postulate that some of the new variants identified in our study are deleterious, either as a monogenic dominant mutation or through a modifier effect, while we cannot exclude the hypothesis that some variants may be very rare benign polymorphisms. The two variants, p.P1583S and p.L4670V were considered as highly probably pathogenic based on the conservation of the amino acid concerned, the absence of a large control population and the damaging effect predicted in silico. The familial studies showed that the
variants segregate with the phenotype but with reduced penetrance. The pathogenic effect of p.A2213S, p.G2367R, p.Y2932H, p.V3219M was more difficult to ascertain. In silico analyses suggest that these variants are possible benign polymorphisms or modifying variants rather than direct disease-causing mutations.

Noticeably, we identified the presence of the two RYR2 SNPs (p.G1885E and p.G1886S) in a compound heterozygous manner in one additional proband, which were previously reported to be associated with ARVC/D (data not shown)\(^24\). However, we also identified this association in a healthy control subject supporting a possible modifier effect of these variants rather than a causal role.

The RYR2 mutations identified so far in ARVC/D were located in the cytoplasmic region of the channel\(^11,22,23\). Similarly, all the genetic variants we identified were also located in the cytoplasmic part of the protein, except the p.L4670V variant, which is located into the transmembrane domain, a hot spot of RYR2 mutations associated with CPVT phenotypes\(^29\). We therefore believe that the molecular analyse of RYR2 in ARVC/D requires the screening of the entire coding region of the gene.

**Phenotype/genotype analysis**

Until now, only few studies reported RYR2 mutations associated with ARVC/D\(^11,22,23\) (ARVC2 locus). In those families, the phenotype linked to RYR2 mutations appeared different from desmosome-related ARVC/D and presented overlapped characteristics with CPVT\(^11\).

Interestingly, in the present study, the phenotype associated with RYR2 variants is similar to desmosome-related ARVC/D. Most probands fulfilled ARVC/D diagnosis based on recent update TFC\(^25\). They exhibited frequent TWI or parietal block on right precordial leads, which are common ARVC/D features absent from the CPVT and the ARVC2 phenotype. All probands displayed ventricular arrhythmias that were predominantly monomorphic with a LBBB morphology, which is a usual feature of ARVC/D, in opposite to the polymorphic
arrhythmias encountered in CPVT or ARVC2. Ventricular effort-induced polymorphic
ventricular arrhythmias are an important diagnosis feature of ARVC2 and CPVT\textsuperscript{19,21}, however
this feature was rarely present in our RYR2-related ARVC/D cohort (supplementary table 1). The p.P1583S carriers exhibited a phenotype closer to ARVC2 with effort-induced ventricular
fibrillation and polymorphic VEc associated with significant RV abnormalities and few
baseline ECG abnormalities. All patients displayed right ventricular cardiomyopathy with
either RV dilatation and/or WMA usually encountered in ARVC/D. The endocardial mapping
of the proband carrying the putative p.L4670V mutation showed a zone of scar tissue within
the inferior RV wall. One proband developed a severe biventricular cardiomyopathy leading
to heart transplant. The explanted heart showed typical histological features of ARVC/D with
extensive fibro-fatty replacement of myocytes.

Role of RYR2 variants in ARVC/D physiopathology

The Ca\textsuperscript{2+} release channel RYR2 plays a central role in cardiac excitation-contraction
coupling\textsuperscript{30}. During the systole, RYR2 massively releases Ca\textsuperscript{2+} from the sarcoplasmic reticulum
(SR) towards the cytoplasm leading to the cardiac contraction. Recently, a mutation affecting
the phospholamban (PLN gene), another protein playing a central role in calcium regulation,
has been described in an ARVC/D and DCM (dilated cardiomyopathy) population\textsuperscript{17}.

Even if the PLN phenotype is possibly particular or overlapping, this observation might
suggest a potential link between [Ca\textsuperscript{2+}] dysregulation and arrhythmogenic cardiomyopathies.
It could be hypothesised that modifications of intracellular calcium homeostasis induced by
RYR2 mutations might alter signaling pathways dependent of intracellular Ca\textsuperscript{2+} homeostasis,
and participate to the pathogenesis of ARVC/D. However, this hypothesis requires further
investigations.

Limits
A limit of this study is the absence of definitive demonstration for the pathogenicity of the
RYR2 variants, because of inconclusive segregation analyses and no available functional
studies. However, one of the variants was previously reported to cause a gain of function\textsuperscript{27,28}. Moreover, the small size of families is quite usual in ARVC/D because of reduced penetrance
and therefore few affected relatives in a family. In addition we performed a careful and
conservative interpretation of the variants.

Another limit concerns the comparison of the rate of RYR2 variants in our cohort and in EVS
cohort while two different methods of sequencing were used, which performance may differ
(error rate, coverage). However, their efficiency in term of error rate seems to be similar\textsuperscript{31} and
the coverage of the coding sequence of RYR2 in EVS was excellent (99.9%). We are therefore
confident with the true difference of RYR2 variants in patients and controls, although the
analysis of a larger cohort of patients with NGS could strengthen our findings.

At last, the ARVC/D probands were selected according to the 1994 Task Force Criteria as this
cohort was prospectively recruited before the publication of the revised TFC.

CONCLUSION
We performed the first systematic screening of the whole RYR2 gene in a well clinically
characterized ARVC/D cohort, after the exclusion of patients with pathological mutations in
five desmosomal genes, and identified putative RYR2 mutations in 9% of ARVC/D probands.
Furthermore, we observed that RYR2 variants were associated with ARVC/D not overlapping
with CPVT phenotype. This observation suggests that RYR2 variants play a role in the genetic
basis of conventional ARVC/D either as disease-causing mutation, or a modifier gene. This
study also support the notion that in case of a missense variant identified in RYR2 in ARVC/D
patient, it is necessary to be particularly cautious in the interpretation of the pathogenicity of
the variant. The screening of multiple genes, including RYR2, in ARVC/D populations in
association with careful phenotype/genotype correlations and functional studies will help to better understand the complex genetic background of this disease.

ACKNOWLEDGMENTS

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van der Zwaag PA, van Rijsingen IA, Asimaki A, et al. Phospholamban R14del mutation in patients diagnosed with dilated cardiomyopathy or arrhythmogenic right


32 Du GG, Sandhu B, Khanna VK, Guo XH, MacLennan DH. Topology of the Ca2+ release channel of skeletal muscle sarcoplasmic reticulum (RyR1). *Proc Natl Acad Sci USA* 2002; 99: 16725-16730.
CLINICAL PERSPECTIVES

To our knowledge, we report here the first systematic screening of the whole RYR2 gene in a large ARVC/D cohort without mutation in desmosomal genes. We identified six rare RYR2 missense variants predominantly associated with a conventional phenotype of ARVC/D. Using the data from the Exome Variant Server database, we estimated the prevalence of rare RYR2 variants to 4% in the general population, which was significantly lower to the 9% observed in our cohort (Fisher test, p=0.0322). The results support the role of RYR2 gene in conventional ARVC/D. This study also highlights the fact that in case of a missense variant identified in RYR2 in ARVC/D patient, it is necessary to be particularly cautious in the interpretation of the pathogenicity of the variant given the prevalence of rare RYR2 variants in the general population. The screening of multiple genes, including RYR2, in ARVC/D populations in association with careful phenotype/genotype correlations and functional studies will help to better understand the complex genetic background of this disease and to improve the management of ARVC/D patients in the future.
FIGURE LEGENDS

Figure 1. Family pedigrees of the patients with a *RYR2* variant.
Square: male, circle: women, white: unaffected individuals after clinical examination or with no known disease, black: affected, grey: borderline (TFC 2010). +/-: heterozygous individuals, -/-: individuals negative. NA: genetic status not available. aSCD: aborted sudden cardiac death

Figure 2. Electrocardiographic characteristics of *RYR2* variants carriers
Proband II:1, family A displayed an unusual intraventricular conduction defect in lead V1 (arrow). Proband II:1 and his sister II:2 from family B and probands from family D and E displayed T-wave inversion in precordial leads V1 to V3. Proband from family F displays an iRBBB and epsilon wave in V1 (arrow) and multifocal wide Vec with LBBB morphology.

Figure 3. Electrophysiological data.
Proband II:1, family B developed spontaneous monomorphic NSVT recorded during the 24-hours ECG monitoring. The electrophysiological study triggered a SVT with LBBB morphology and superior axis (left), close to the spontaneous ventricular ectopies morphology (right). EP study performed on proband II:4, family D triggered NSVT with LBBB morphology.

Figure 4. Histological data.
Histological characterisation of the proband from family F. The figure showed typical fibrofatty replacement in the right ventricle anterior wall of explanted heart (haematoxylin and eosin staining, x20 and x100 magnification)
Figure 5. Location and conservation of the *RYR2* variants identified.

(A) Location: all missense variants are located within the intra-cytoplasmic part or the protein, at the exception of the p.L4670V, which is located within the trans-membranous part of the protein. (B) The conservation of the mutated residues 1583 and 4670 indicate that they are highly conserved across human isoforms and species.
Table 1. Clinical characteristics of probands RYR2 mutation carriers.

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Mutation</th>
<th>Age</th>
<th>Familial status</th>
<th>Origin</th>
<th>Symptoms</th>
<th>Familial history</th>
<th>ARVC in first degree relative</th>
<th>Ventricular arrhythmia</th>
<th>RV abnormalities</th>
<th>LV abnormalities</th>
<th>ECG</th>
<th>SAECG</th>
<th>Histology</th>
<th>Treatment</th>
<th>Clinical status according to 2010 TFDC</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>II:1</td>
<td>p.P1583S</td>
<td>22</td>
<td>Pb Europe</td>
<td>Aborted SCD</td>
<td>none</td>
<td>no</td>
<td>Yes*</td>
<td>Dilated RV, apical trabeculations, infero-lateral dyskinesia and akinesia of antero lateral RVOT (echo, MRI, angiography)**</td>
<td>Mild LV dilatation and infero-basal hypokinesia</td>
<td>3/3*</td>
<td>NA</td>
<td>BB, ACEI, ICD</td>
<td>Affected</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:3</td>
<td>p.P1583S</td>
<td>17</td>
<td>R Europe</td>
<td>none</td>
<td>Yes</td>
<td>Yes** none</td>
<td>Mild RV dilatation on echocardiography with lowered RV FAC 27% and dysynchronous RV contraction with apico-lateral hypokinesia on cardiac-MRI (echo/MRI)</td>
<td>Terminal QRS duration &gt; 55ms in V1*</td>
<td>none</td>
<td>0/3</td>
<td>NA</td>
<td>none</td>
<td>Borderline</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>I:1</td>
<td>p.P1583S</td>
<td>60</td>
<td>R Europe</td>
<td>none</td>
<td>none</td>
<td>Yes** Yes*</td>
<td>RV apical akinesia associated with RV enlargement (RVOT measured at 34 mm PSAX view) (echo, angiography)*</td>
<td>LVEF = 55%</td>
<td>TWI V1-3**</td>
<td>0/3</td>
<td>NA</td>
<td>BB</td>
<td>Affected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>II:1</td>
<td>p.L4670V</td>
<td>43</td>
<td>Pb Europe</td>
<td>none</td>
<td>none</td>
<td>no</td>
<td>Yes*</td>
<td>Mild RV dilatation (RV end-diastolic volume = 109 ml/m²) and global hypokinesia RV EF 36% (echo/MRI)</td>
<td>None (echo,MRI, scintigraphy)</td>
<td>TWI V1-4**</td>
<td>1/3*</td>
<td>NA</td>
<td>BB</td>
<td>Affected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:2</td>
<td>p.L4670V</td>
<td>41</td>
<td>R Europe</td>
<td>none</td>
<td>Yes</td>
<td>Yes** none</td>
<td>None (echo,MRI, scintigraphy)</td>
<td>TWI V1-3**</td>
<td>0/3</td>
<td>NA</td>
<td>none</td>
<td>Affected</td>
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<tr>
<td></td>
<td>I:2</td>
<td>p.L4670V</td>
<td>70</td>
<td>R Europe</td>
<td>none</td>
<td>Yes</td>
<td>Yes** none</td>
<td>None (echo, MRI, scintigraphy)</td>
<td>Normal</td>
<td>2/3*</td>
<td>NA</td>
<td>none</td>
<td>borderline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>II:3</td>
<td>p.G2367R</td>
<td>59</td>
<td>Pb North Africa</td>
<td>Presyncope</td>
<td>none</td>
<td>no</td>
<td>Yes*</td>
<td>WMA with apical and RVOT akinesia, RV LGE* (echo, MRI, scintigraphy)</td>
<td>None</td>
<td>RBBB; QRS V1&gt;60ms,5ms</td>
<td>3/3</td>
<td>NA</td>
<td>BB, amiodarone, ACEI, ICD</td>
<td>Borderline</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>II:4</td>
<td>p.A2213S</td>
<td>35</td>
<td>Pb Europe</td>
<td>Palpitations syncope</td>
<td>none</td>
<td>no</td>
<td>Yes*</td>
<td>RV dilatation (RVOT at 50 mm in PSAX view)</td>
<td>None</td>
<td>TWI V1-3**</td>
<td>1/3*</td>
<td>NA</td>
<td>BB, ICD</td>
<td>Affected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:4</td>
<td>p.A2213S</td>
<td>12</td>
<td>R Europe</td>
<td>none</td>
<td>Yes</td>
<td>Yes** none</td>
<td>None (echo)</td>
<td>None</td>
<td>NI</td>
<td>0/3</td>
<td>NA</td>
<td>none</td>
<td>Unaffected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>II:2</td>
<td>p.V3219M</td>
<td>41</td>
<td>Pb Europe</td>
<td>Presyncope</td>
<td>none</td>
<td>no</td>
<td>Yes*</td>
<td>WMA with dyskinesia and RV aneurysm (echo, MRI)</td>
<td>None</td>
<td>TWI V1-3**</td>
<td>0/3</td>
<td>NA</td>
<td>BB</td>
<td>borderline</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>II:4</td>
<td>p.Y2932H</td>
<td>39</td>
<td>Pb Europe</td>
<td>Palpitations dyspnea</td>
<td>none</td>
<td>no</td>
<td>Yes*</td>
<td>Major RV dilatation, antero akinesia, anterior akinesia and infero-apical severe hypokinesia, trabeculations (echo, angiography)**</td>
<td>LVEF = 45%</td>
<td>Mild LV dilatation</td>
<td>0/3</td>
<td>↑**</td>
<td>Amodarone, BB, ACEI, ICD, Heart Tx</td>
<td>Affected</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:3</td>
<td>p.Y2932H</td>
<td>41</td>
<td>R Europe</td>
<td>none</td>
<td>Yes</td>
<td>Yes** none</td>
<td>None (echo)</td>
<td>None</td>
<td>NI</td>
<td>0/3</td>
<td>NA</td>
<td>none</td>
<td>Unaffected</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II:2</td>
<td>p.Y2932H</td>
<td>42</td>
<td>R Europe</td>
<td>none</td>
<td>Yes</td>
<td>Yes** none</td>
<td>None (echo)</td>
<td>None</td>
<td>NI</td>
<td>0/3</td>
<td>NA</td>
<td>none</td>
<td>Unaffected</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ACEI: angiotensin conversion enzyme inhibitor; AF: atrial fibrillation; BB: beta-blockers; Echo: echocardiography; EF: ejection fraction; FAC: fractional area change; Heart Tx: heart transplantation; ICD: implantable cardiac defibrillator; LGE: presence of late-gadolinium enhancement; LV: left ventricle; NA: non-available data; Pb: proband (all fulfilled 1994 diagnostic criteria available at enrolment); PSAX: para-sternal short axis; R: relative; RV: right ventricle; RBBB: right bundle branch block; SCD: sudden cardiac death; SAECG: signal average ECG; RVOT: right ventricle outflow tract; TWI: T-wave inversion; WMA: wall motion abnormalities; *: min or criteria according to the 2010 Task Force Diagnosis Criteria (TFDC); **: major criteria; ↑: fibrofatty replacement.
Table 2: Predicted alterations of nonsynonymous changes observed.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Mutation</th>
<th>Location</th>
<th>Conservation</th>
<th>Prediction from bioinformatic tools</th>
<th>Frequency in variant database*</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>c.4747C&gt;T</td>
<td>Cytoplasmic</td>
<td>yes</td>
<td>Intolerant</td>
<td>Probably damaging</td>
</tr>
<tr>
<td></td>
<td>p.P1583S</td>
<td></td>
<td></td>
<td></td>
<td>Disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Not reported in EVS &amp; 1000 genomes (but reported in NCBI (frequency 1/24 000))</td>
</tr>
<tr>
<td>43</td>
<td>c.6637G&gt;T</td>
<td>Cytoplasmic</td>
<td>no</td>
<td>Intolerant</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td></td>
<td>p.A2213S</td>
<td></td>
<td></td>
<td></td>
<td>Disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Not reported</td>
</tr>
<tr>
<td>46</td>
<td>c.7099G&gt;A</td>
<td>Cytoplasmic</td>
<td>no</td>
<td>Tolerant</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td></td>
<td>p.G2367R</td>
<td></td>
<td></td>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.008%</td>
</tr>
<tr>
<td>60</td>
<td>c.8794T&gt;C</td>
<td>Cytoplasmic</td>
<td>no</td>
<td>Tolerant</td>
<td>Probably damaging</td>
</tr>
<tr>
<td></td>
<td>p.Y2932H</td>
<td></td>
<td></td>
<td></td>
<td>Neutral</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Not reported</td>
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<tr>
<td>68</td>
<td>c.9655G&gt;A</td>
<td>Cytoplasmic</td>
<td>no</td>
<td>Tolerant</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td></td>
<td>p.V3219M</td>
<td></td>
<td></td>
<td></td>
<td>Disease</td>
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</tr>
<tr>
<td>97</td>
<td>c.14008C&gt;G</td>
<td>Transmembrane domain</td>
<td>yes</td>
<td>Intolerant</td>
<td>Probably damaging</td>
</tr>
<tr>
<td></td>
<td>p.L4670V</td>
<td></td>
<td></td>
<td></td>
<td>Disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Not reported</td>
</tr>
</tbody>
</table>

*Including NCBI, EVS (exome variant server) and 1000 genomes databases
Figure 2
Electrocardiographic recording

- Fam A
  - Proband (patient II:1)
  - Relative (patient II:3)

- Fam B
  - Proband (patient II:1)
  - Relative (patient II:2)

- Fam D
  - Proband (patient II:4)

- Fam E
  - Proband (patient II:2)

- Fam F
  - Proband (patient II:4)
Figure 3

Proband (patient II:1), family B

Electrophysiological study

Proband (patient II:4), family D

Electrophysiological study

24-hours ECG monitoring
Histological characterisation of the proband (patient II:4), fam F.
Click here to download Supplementary Material: supplementary_table1.doc