Received: 9 December 2021 | Revised: 13 May 2022 | Accepted: 25 May 2022

DOI: 10.1002/humu.24436

RESEARCH ARTICLE

Human Mutation HGVS HUMAN GENOME VARIATION SOCIETY WILEY

Burden of rare variants in arrhythmogenic cardiomyopathy with right dominant form-associated genes provides new insights for molecular diagnosis and clinical management

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Funding information
Julien Barc is supported by the CRITERIA project funded by the French Federation of Cardiology.; Julien Barc is supported by the research program Etoiles montantes des Pays de la Loire REGIOCARD RPH081-U1087-REG-PDL; Fondation de l'Avenir et la Ligue Nationale de Basket, Grant/Award Number: RAK20104NNA

Abstract

Arrhythmogenic cardiomyopathy with right dominant form (ACR) is a rare heritable cardiac cardiomyopathy disorder associated with sudden cardiac death. Pathogenic variants (PVs) in desmosomal genes have been causally related to ACR in 40% of cases. Other genes encoding nondesmosomal proteins have been described in ACR, but their contribution in this pathology is still debated. A panel of 71 genes associated with inherited cardiopathies was screened in an ACR population of 172 probands and 856 individuals from the general population. PVs and uncertain significance variants (VUS) have been identified in 36% and 18.6% of patients, respectively. Among the cardiopathy-associated genes, burden tests show a significant enrichment in PV and VUS only for desmosomal genes

PKP2 (plakophilin-2), *DSP* (desmoplakin), *DSC2* (desmocollin-2), and *DSG2* (desmoglein-2). Importantly, VUS may account for 15% of ACR cases and should then be considered for molecular diagnosis. Among the other genes, no evidence of enrichment was detected, suggesting an extreme caution in the interpretation of these genetic variations without associated functional or segregation data. Genotype–phenotype correlation points to (1) a more severe and earlier onset of the disease in PV and VUS carriers, underlying the importance to carry out presymptomatic diagnosis in relatives and (2) to a more prevalent left ventricular dysfunction in *DSP* variant carriers.

KEYWORDS
arrhythmogenic cardiomyopathy, burden tests, molecular diagnosis, next-generation sequencing

1 | INTRODUCTION

Arrhythmogenic cardiomyopathy with right dominant form (ACR) is an autosomal dominant myocardial disease affecting mainly young people between the ages of 20 and 40 years (Wang et al., 2019). Autosomal recessive forms are described and associated with wooly hair and palmoplantar keratoderma (Naxos disease) (Protonotarios & Tsatsopoulou, 2004). In this arrhythmogenic cardiomyopathy, cardiomyocytes are progressively replaced by fibrosis and adipose tissue, leading to arrhythmic events. Sudden death can be the first symptom, especially in young athletes (Corrado et al., 2003; Marcus et al., 1982). The early diagnosis of this disease is mandatory to provide appropriate clinical management with the reduction of mortality and prevention of disease progression (Towbin et al., 2019). Diagnostic criteria are standardized in a Task Force (Marcus et al., 2010), requiring a combination of structural, electrocardiographic, rhythmic, histological, and genetic factors. About 50% of affected patients carry a variant in genes involved in ACR (Groeneweg et al., 2015). Genes encoding desmosomal proteins are the most frequently mutated: *PKP2* (plakophilin-2), *DSC2* (desmocollin-2), *DSG2* (desmoglein-2), *DSP* (desmoplakin), and *JUP* (junction plakoglobin). These proteins form a functional unit with adherens junction and gap junction and play a key role in cell-to-cell interaction and action potential propagation. Eleven other genes are associated with this disorder and encode nondesmosomal proteins: adherens junctions (*CTNNA3*, *CDH2*), nuclear envelope (*LMNA*, *TMEM43*), intermediate filaments (*DES*), sarcomere (*TTN*), calcium-regulating proteins (*RYR2*, *PLN*), cardiac sodium channel (*SCN5A*), growth factor (*TGFβ3*), and transcription factor (*TP63*). According to the study by van Lint et al. (2019), 6.8% of probands carry pathogenic variants (PVs) and likely pathogenic variants (LPVs) in nondesmosomal genes. However, the implication of these nondesmosomal genes is still much debated due to low penetrance and variable expressivity (Gandjbakhch et al., 2018).

Whole-genome and whole-exome exploration from thousands of individuals in the general population revealed the high variability of the human genome, and in particular, the abundance of rare variants (<0.1%), modifying the protein sequence (Karczewski et al., 2019) with no clinical consequences, demonstrating that protein sequence changed even coupled to a low allele frequency is not sufficient to classify the variant as pathogenic. Variant interpretation is therefore challenging and standardized procedures and tools are required to distinguish PVs from physiological genetic polymorphism (Kapplinger et al., 2011). In 2015, the American College of Medical Genetics and the Association for Molecular Pathology (ACMG-AMP) (Richards et al., 2015) published international guidelines to classify variants in five classes from PVs (Class 5) to benign variants (Class 1). Epidemiologic, functional, structural, and clinical criteria are used and different weights are assigned for each of these criteria. The combination of these criteria and their respective weights allow to assign a pathogenicity class for each variant. Nevertheless, Amendola et al. (2016) demonstrated that following these recommendations is time-consuming and remains a subjective process. Bioinformatic tools exist to semiautomate these guidelines (InterVar; Li & Wang, 2017), but they have their own limitations and remains far from being perfect (eVAI, Nicora et al., 2018; CardioClassifier, Whiffin et al., 2018).

PV or LPV in genes associated with ACR is a major criterion in the Task Force diagnostic work-up, and the way in which the variant is classified will therefore influence not only the result of the genetic analysis but also the patient's diagnosis, which reinforces the importance of an accurate classification that remains the most delicate step of the genetic diagnosis. The relevance of nondesmosomal genes associated with ACR is debated (James et al., 2021; Patel et al., 2020; Rehm et al., 2015), questioning the selection of genes to include in ACR patients' molecular diagnosis. Moreover, the proportion of uncertain significance variant (VUS) in ACR cases (James et al., 2020) leads to questioning the number of patients deprived of molecular diagnostic and preventive action for their relatives. Here, we assess the prevalence of genes associated with ACR in a large population of 172 ACR unrelated patients. We then study the relevance of genes involved in ACR by comparing the numbers of PV,

LPV, and VUS between ACR patient set and a control population of 856 individuals. We evaluate the part of genetic variants annotated as the uncertain significance and that could actually be associated with the disease and then be integrated into the gene panel for a molecular diagnosis. We also investigate the correlation between genetic variants and clinical characteristics at the diagnosis and during the follow-up of patients.

2 | METHODS

2.1 | Patient and control set description

A set of 172 index cases was included in this study. The diagnosis of ACR was performed by an expert cardiologist from the referral center "Le centre de référence des troubles du rythme cardiaque héréditaires ou rares de l'Ouest," according to the Task Force criteria (Marcus et al., 2010). They were recruited by the following French centers: Angers, Angoulême, Annecy, Bayonne, Bordeaux, Brest, La Rochelle, Le Mans, Lille, Montpellier, Nantes, Rennes, La Réunion, Toulouse, and Tours. The right or left ventricular dysfunction is defined by ventricular ejection fraction ≤40% based on magnetic resonance imaging (MRI) and corresponds to a major criteria. The ventricular ejection fraction between >40% and ≤45% based on MRI imaging corresponds to a minor criteria. Ventricular tachycardia (VT) was defined by wide QRS tachycardia with a frequency >120/min and a dedicated electrocardiographic pattern. The sustained and the nonsustained VT are grouped under "VT" characterized by left bundle-branch morphology with a superior axis.

The control population comprises 856 individuals from the general population with no history of cardiac arrhythmia. The control population used was from the consortium FranceGenRef, which contained unrelated healthy blood donors from three different studies (the PREGO cohort [www.vacarme-project.org], the GAZEL cohort [www.gazel.inserm.fr/en], and 50 healthy subjects from the Finistère area). All individuals signed informed consent for genetic studies at the time they were enrolled. Corresponding whole-genome sequencing individual data with an average depth of 30× with 10× coverage for 99.8% of the genome have been used as control.

This study was conducted according to French Guidelines for clinical and genetic research. Informed written consent was obtained from each patient who agreed to participate in the genetic study.

2.2 | ACR genes sequencing in cases

Genomic DNA was extracted from peripheral blood lymphocytes by standard protocols. The custom design is based on HaloPlex™ technology (Agilent Technologies) to perform high-throughput sequencing of the coding regions of 71 genes (Supporting Information: Table S1) previously linked to arrhythmia and cardiomyopathies including 11 genes associated with ACR (*DSC2*, *DSG2*, *DSP*, *JUP*, *LMNA*, *PKP2*, *PLN*, *RYR2*, *SCN5A*, *TGFβ3*, and *TMEM43*). Capture and library preparation was performed following the manufacturer's instruction. One hundred and fifty base pair paired-end Illumina sequencing on HiSeq 1500 was performed.

2.3 | Detection of rare genetic variants

Raw sequence reads are aligned to the human reference genome (GRCh37/hg19) using BWA-MEM (v0.7.15-r1142-dirty). Variants are called for each sample by GATK (v3.6) and annotated with SnpEff (v4.3T). Only positions with coverage greater than 10× for 90% of patients are further investigated. Variants are considered rare if the allele frequency was <0.1% compared to gnomAD data, in genome and exome from non-Finish European population (gnomAD v2.1.1 last consulted in October 2019). Only rare variants with genotype quality higher than 100 are selected. Rare variants are selected with a quality index higher than 100. Exonic variants and intron and splicing variants with ±10 bp are retained.

2.4 | Assessment of pathogenicity of variants

According to ACMG Guidelines (Richards et al., 2015), the pathogenicity of variants was assessed by two bioinformatic tools: InterVar (Li & Wang, 2017) (downloaded in January 2019 and last used in October 2019) and eVAI (Nicora et al., 2018) (downloaded in January 2019 and last used in October 2019). The following criteria of InterVar were modified (BA1, BS1, PM2, and PM5) or inactivated (BS2 and PS4) due to the characteristics of ACR (Supporting Information: Table S2). A variant is considered pathogenic or benign when at least one of the two tools predicts it to be pathogenic or benign, respectively. A variant is considered likely pathogenic or likely benign when at least one of the two tools predicts likely pathogenic or likely benign, respectively. A variant is considered uncertain significance when both tools predict uncertain significance simultaneously. With eVAI, the indication can be chosen and we then selected arrhythmogenic cardiomyopathy. In silico tools were used to assess the pathogenicity of variants: Grantham score (amino acid conservation), ADA and RF score (splicing impact), and CADD phred score (v1.4) (missense variants). The mean score of these different tools was compared across the class of variants in the patient set and control set for each gene with Student's *t* test. A $p < 0.05$ shows a significant difference.

2.5 | Validation of the ACMG bioinformatics tools: eVAI and InterVar

Fifty-two variants from 52 patients with long QT syndrome were considered to evaluate the performance of the two bioinformatic tools on five genes (*SCN5A*, *KCNH2*, *KCNQ1*, *CACNA1C*, and *KCNJ2*). These variants were classified according to ACMG's classification by the molecular diagnostic laboratory. This validation is based on predicted PV, LPV, and VUS because benign variants are not

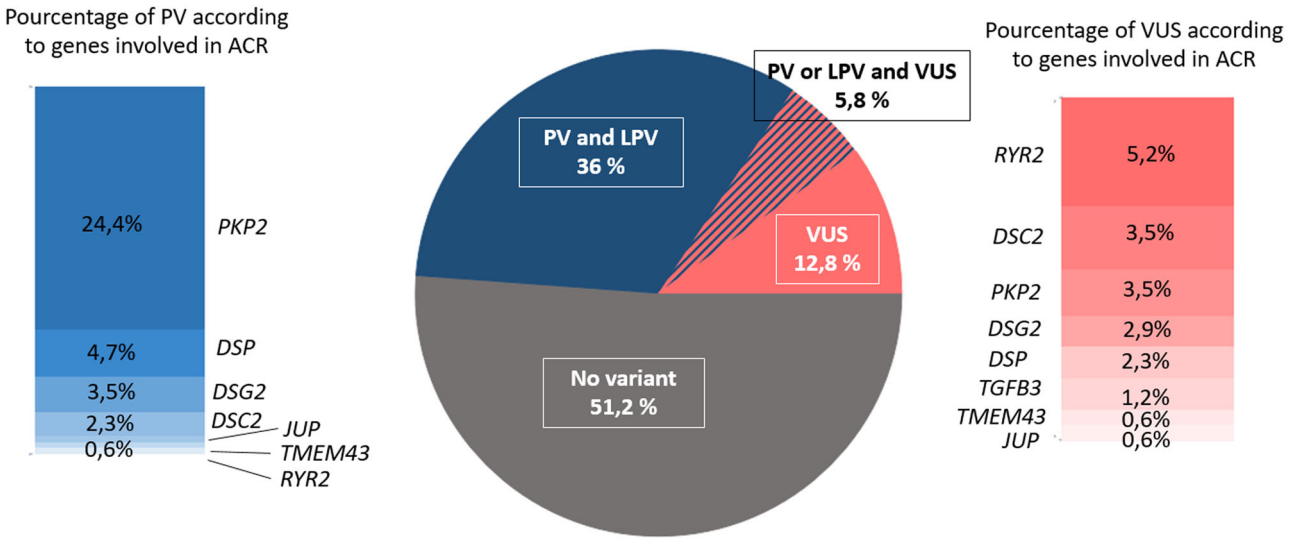

FIGURE 1 Prevalence of genes involved in ACR in patient set according to the class of pathogenicity of variants. Gray: Patients with a negative genotype; blue: patients with PV or LPV; pink: patients with VUS. ACR, arrhythmogenic cardiomyopathy with right dominant form; LPV, likely pathogenic variants; PV, pathogenic variants; VUS, uncertain significance variants.

rendered by the molecular diagnostic laboratory. Ninety-four percent (49 variants among 52 tested variants) of the classification of variants with InterVar and eVAI is consistent with the classification of the diagnostic laboratory. The three nonconcording variants are classified as likely pathogenic or pathogenic by the diagnostic laboratory and uncertain significance by the tools. These variants have already been found in patients with long QT syndrome of "Le centre de référence des troubles du rythme cardiaque héréditaires ou rares de l'Ouest" but not published in the literature.

2.6 | Burden tests

Burden tests were carried out to compare the proportion of VUS, LPV, and PV between 172 ACR cases and 856 controls. Two tests were performed for each gene: CAST (Cohort Allelic Sums Test) and SKAT-O (Optimized SKAT) adjusted. Rare variant pathogenicity was assessed with the biocomputing tools eVAI (Nicora et al., 2018) and InterVar (Li & Wang, 2017). Within a gene, the PV, LPV, and VUS are merged for each individual. For each gene, we compared the number of individuals who carry ≥1 PV/LPV or VUS between the AC population and the control population. Bonferonni correction was applied. A p value less than 0.0045 was considered as suggestive of association regarding the 11 tests performed.

2.7 | Descriptive statistical analysis

Student's t test was used to compare the mean age at diagnosis between patient and control sets. A Fisher's exact test was performed to compare the proportion of patients and controls for clinical characteristics at diagnosis and during follow-up.

3 | RESULTS

3.1 | ACR patient set description and sequencing features

One hundred and seventy-two probands with a diagnosis of ACR according to the Task Force criteria (Supporting Information: Table S3) have been included in this study between 2010 and 2014. The mean age at diagnosis was 39.2 ± 15.5 years (13:70) (minimum:maximum). The sex ratio is 130 males to 42 females (3:1). One hundred and forty-eight cases (86%) experienced a rhythmic event, including 14 resuscitated cardiac arrests, 108 VTs, 39 ventricular premature beats, and 1 atrial fibrillation at diagnosis. Patients were followed up between 1 and 41 years with an average of 12.2 ± 8.3 years [mean ± standard deviation]. One individual presented with Naxos disease.

DNA from 172 patients was sequenced for 71 genes (Supporting Information: Table S1) previously linked to arrhythmia and cardiomyopathies, including 11 genes associated with ACR (*DSC2*, *DSG2*, *DSP*, *JUP*, *LMNA*, *PKP2*, *PLN*, *RYR2*, *SCN5A*, *TGFβ3*, and *TMEM43*). The mean sequencing depth was 542X per sample with a mean coverage of 98.7% of the targeted regions covered at least 10 times (Coding Exons + untranslated region [UTRs] + 5′-UTR + 3′-UTR with 50 bp from 3′ end and 50 bp from 5′ end).

3.2 | Prevalence of variants among genes involved in ACR according to their pathogenicity class

The prevalence of 11 genes involved in the ACR population is represented in Figure 1.

TABLE 1 Burden tests results for 11 genes associated with ACR

Genes	Pathogenic/likely pathogenic variants				Uncertain significance variants			
	ACR patient set ($n = 172$)	Control population ($n = 856$)	p value CAST	p value SKAT-O	ACR patient set ($n = 172$)	Control population ($n = 856$)	p value CAST	p value SKAT-O
PKP2	24.4% (42)	0	2.42×10^{-35}*	1.13×10^{-39}*	3.5% (6)	0.4% (3)	1.09×10^{-3}*	0.032*
DSP	4.7% (8)	0	5.35×10^{-7}*	1.13×10^{-6}*	2.3% (4)	1.4% (12)	0.325	0.940
DSG2	3.5% (6)	0	2.04×10^{-5}*	1.13×10^{-5}*	2.9% (5)	0.4% (3)	4.52×10^{-3}*	0.093
DSC2	2.3% (4)	0	7.61×10^{-4}*	2.48×10^{-4}*	3.5% (6)	0.1% (1)	5.23×10^{-4}*	4.87×10^{-3}*
JUP	0.6% (1)	0	0.167	0.029*	0.6% (1)	1.2% (10)	0.702	0.319
SCN5A	0	0.1% (1)	1	0.654	0	0	-	-
TMEM43	0.6% (1)	0	0.167	0.029*	0.6% (1)	0.6% (5)	1	0.780
RYR2	0.6% (1)	0.2% (2)	0.423	0.775	5.2% (9)	3.7% (32)	0.391	0.573
PLN	0	0	-	-	0	0	-	-
LMNA	0	0	-	-	0	0	-	-
TGFβ3	0	0	-	-	1.2% (2)	0.6% (5)	0.332	0.821

Note: The proportion of ACR cases or controls (in %) carrying at least one pathogenic/likely pathogenic variant or uncertain significance variants in each gene is shown.

Abbreviations: ACR, arrhythmogenic cardiomyopathy with right dominant form; CAST, Cohort Allelic Sums Test; SKAT-O, optimized SKAT.

*Significant p value.

Seventy-nine PV, LPV, or VUS in genes associated with ACR have been identified in 48.8% (84 patients) of patients. ACR PV and LPV (Supporting Information: Table S4) have been found in 36% of patients (62 index cases, 63 PVs and LPVs); 24.4% of patients (42 cases) carry a PV in *PKP2*, followed in a smaller proportion by four other desmosomal genes such as *DSP*, *DSG2*, *DSC2*, and *JUP* with 4.7% (8 patients), 3.5% (6 patients), 2.3% (4 patients), 0.6% (1 patient), respectively (Figure 1). One PV in *TMEM43* and *RYR2*, that is, in 0.6% of patients (1 patient), respectively. VUS (Supporting Information: Table S5) are found in 18.6% of patients (32 index cases, 34 VUS); 5.2% of patients (9 cases) carry a VUS in *RYR2*, followed by desmosomal genes *DSC2*, *PKP2*, and *DSG2* with 3.5% (6 patients), 3.5% (6 patients), and 2.9% (5 patients), respectively (Figure 1).

In our ACR population, 7.3% of patients (13 cases) are carriers of double variants in accordance with previous reports (Bauce et al., 2010). One patient carries two PVs in *PKP2* and *DSC2*; 5.6% of patients (10 patients) are carriers of one VUS associated with one PV only in desmosomal genes *PKP2*, *DSC2*, *DSG2*, and *DSP*. Furthermore, 1.7% of patients (2 cases) have two VUS in genes *PKP2*, *RYR2*, and *DSG2*.

Among the 11 genes of the panel associated with ACR, only seven genes (*PKP2*, *DSP*, *DSG2*, *DSC2*, *JUP*, *TMEM43*, and *RYR2*) present PV and LPV. Predominantly, these are genes encoding for the desmosomal complex (*PKP2*, *DSC2*, *DSG2*, *JUP*, and *DSP*). The distribution of VUS shows genes encoding for desmosomal proteins and also for calcium-regulating proteins. No PV, LPV, and VUS were found in *PLN* and *LMNA* genes. Similarly, none of the other genes in the panel associated with cardiomyopathies or arrhythmia (Supporting Information: Data) present PV or LPV in our patient set.

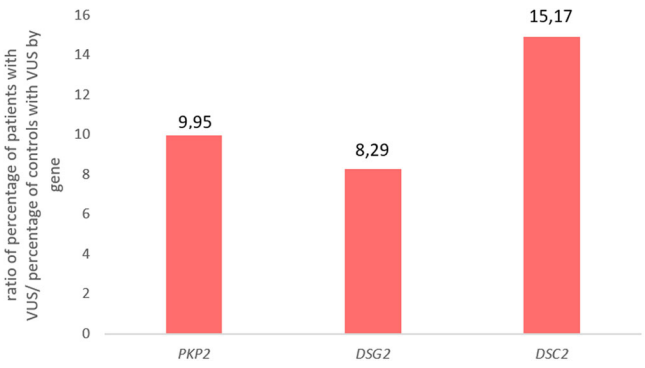

FIGURE 2 Ratio of the percentage of patients with uncertain significance variants (VUS)/percentage of controls with VUS by genes. *PKP2*, *DSG2*, and *DSC2* are significantly enriched in VUS in arrhythmogenic cardiomyopathy with right dominant form cases compared to controls.

3.3 | Burden tests according to the pathogenicity class among genes involved in ACR

We identified a significant enrichment in PV and LPV in *PKP2*, *DSP*, *DSC2*, and *DSG2* in ACR patients with the absence of variants within the control population (Table 1). Interestingly, we uncovered a significant enrichment in VUS in *PKP2*, *DSC2*, and *DSG2* with 8.3–14.9 times more variants in ACR patients compared to controls (Figure 2). VUS are similarly distributed among other ACR-associated genes between ACR patients and the control population (Table 1). The 60 additional genes of the design associated with

cardiomyopathies or arrhythmias do not show a significant enrichment in PV and LPV nor in VUS in ACR cases compared to control individuals (Supporting Information: Table).

3.4 | Assessment of pathogenicity of VUS

To determine whether VUS found in cases could be discriminated from those found in controls, the following criteria were compared: amino acid conservation, prediction in silico scores, and protein location. No hotspot mutation has been identified for these variants across the protein sequences of these two genes (Supporting Information: Figures S1 and S2, with http://lindenb.github.io/pages/uniprot/paintsvg.html). However, for DSC2, VUS in controls are all located in the cytoplasmic domain, whereas in cases they are located preferentially in the extracellular cadherin-binding domain (Supporting Information: Figure S3). A variant found in both cases and controls is located in the cytoplasmic domain. Due to the low number of VUS, the amino acid conservation score or the CADD score could not be tested statistically.

3.5 | Correlation between genotype and phenotype in the ACR patient set

Clinical characteristics of ACR patients between variants carriers and noncarriers were compared. Patients with variants (PV, LPV, and VUS) were diagnosed earlier (36.8 ± 15.8 years old [mean ± standard deviation]) than patients without variants (42.7 ± 14.8 years old; $p = 0.018$). Of note, there is no difference in onset age between carriers of PV or LPV and carriers of VUS ($p = 0.531$). When we specifically considered the age of the diagnosis group between 15 and 24 years old, we found a threefold number of patients with a variant (31.2%) compared to patients without a variant (12.6%) ($p = 0.005$) (Figure 3).

The number of patients with right ventricular dysfunction is significantly greater among patients with variants compared to patients without variants ($p = 0.016$). Likewise, the number of patients with VT ($n = 108$) at diagnosis is significantly greater among patients with variants (including 75% of patients with PV and LPV) compared to patients without variants ($p = 0.012$). Among patients with VT, the gene distribution of ACR-associated genes (*PKP2*: 62.3%; *DSP*: 11.5%; *DSC2*: 4.9%; *DSG2*: 6.6%; *TMEM43*: 3.3%; *JUP*: 3.3%; *RYR2*: 6.6%; *TGFB3*: 1.5%) is similar to the gene distribution in the whole ACR patient population.

Patients with more than one variant (PV or LPV in combination with a VUS in 12 cases out of 13) had no significance at an earlier age of onset compared with patient carriers with one variant ($p = 0.968$). These same patients are no more at risk of arrhythmic events ($p = 1$), and no more damage to right ventricular function ($p = 1$) and left ventricular function (0.383). They do not appear to have a more severe phenotype in our patient set.

Variants in *DSP* gene seem to be predominantly involved in left rather than right ventricular dysfunction ($p = 0.030$; Table 2). During follow-up, all patients with cardiac arrest, syncope, or VT presented with a PV in *PKP2*. Similarly, 7 of 13 patients with heart failure have a PV or VUS in *PKP2* in accordance with previous reports (Vischer et al., 2019).

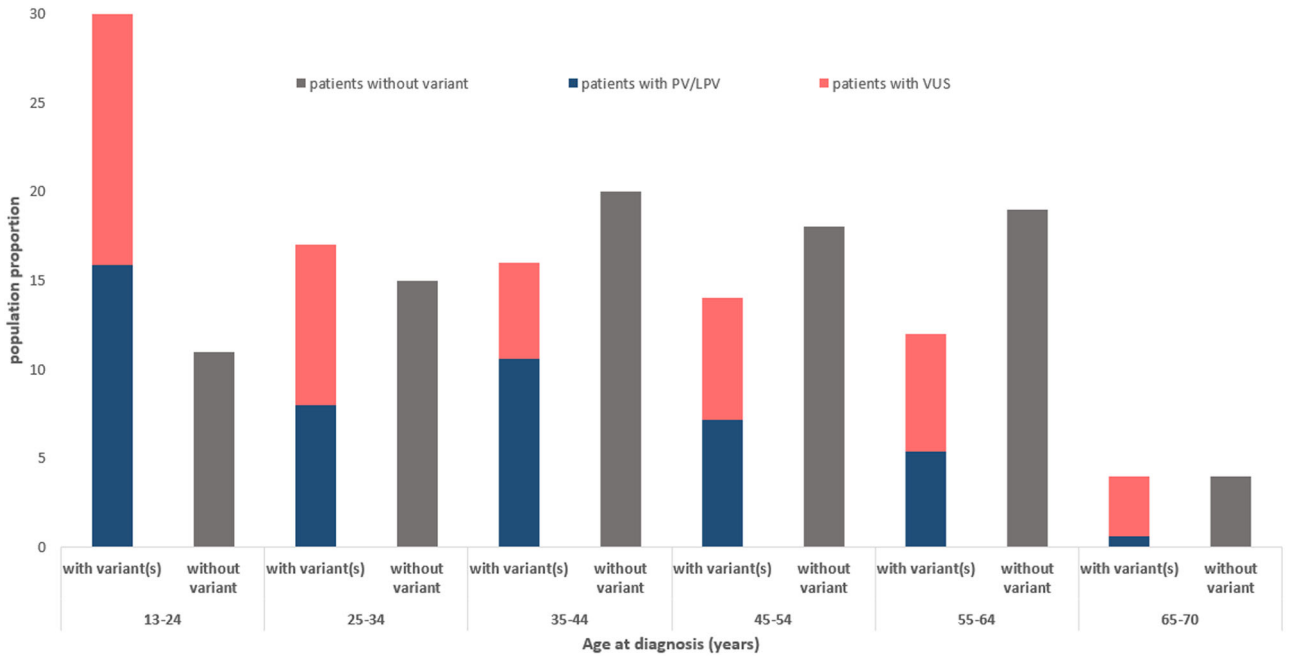

FIGURE 3 Population proportion according to the age of diagnosis and the presence and the class of variants. Gray: Patients with a negative genotype; blue: patients with PV or LPV; pink: patients with VUS. ACR, arrhythmogenic cardiomyopathy with right dominant form; LPV, likely pathogenic variants; PV, pathogenic variants; VUS, uncertain significance variants.

TABLE 2 Proportion of genes with patients with left ventricular damage or right ventricular damage

Genes	Patients with variant: Left ventricular damage ($n = 12$) LVEF < 45%	Patients with variant: Right ventricular damage ($n = 43$) RVEF < 45%	p value
PKP2	41.7% (5)	58.1% (25)	0.345
DSP	41.7% (5)	11.6% (5)	0.030*
DSG2	None	7% (3)	1
DSC2	16.7% (2)	7% (3)	0.298

Abbreviations: LVEF, left ventricular ejection fraction; RVEF, right ventricular ejection fraction.

*Significant p value.

Clinical management of the patient may be personalized since a predominance of left ventricular dysfunction is observed in DSP variant carriers, while only in PKP2 carriers present an arrhythmic event during follow-up in our study.

4 | DISCUSSION

The recent evolution in the next-generation sequencing technology allowed the scientific genetics community as well as the molecular diagnostic departments to change gear in the screening of gene panels, exome or even whole genome with an increased throughput, and decreasing costs. The exploration of individuals from the general population revealed the high polymorphism of the genome including in the rare variant category (Lek et al., 2016). This observation raises the question of the pathogenicity of the genetic variation uncovered in patients and the relevance of variants and genes so far associated with Mendelian diseases. To evaluate variant pathogenicity, the ACMG proposes a classification.

Based on a large cohort of 172 ACR probands, we applied the guidelines proposed by the ACMG to evaluate the level of involvement of 11 genes previously associated with ACR and establish their prevalence and relevance regarding the molecular diagnostic. We also evaluate the involvement of VUS, which has so far been useless in molecular diagnosis. We further investigate the relationship between gene mutations, right and/or left ventricular dysfunction, and ventricular arrhythmia.

In accordance with international experts (ClinGen; Rehm et al., 2015) and recent studies (James et al., 2020), we showed that variants are predominantly found in desmosomal genes explaining about 49% of cases, while variants in genes such as *TMEM43*, *RYR2*, and *TGFβ3* are found in only 7% of cases and correspond mainly to VUS.

Among the PV, 89% of variants (56/63 variants) are nonsense, splicing, or frameshift variants. The loss-of-function mechanism is a pathogenic very strong (PVS1) argument and leads to PV or LPV with regard to the ACMG classification. On the contrary, 76% of VUS (26/34) are missense variants and the remaining VUS are six splice region variants and one disruptive in-frame deletion. Burden tests demonstrate a significant enrichment in PV or LPV for *PKP2*, *DSP*, *DSG2*, and *DSC2* genes, as well as in VUS, for the desmosomal genes *PKP2*, *DSG2*, and *DSC2* in patient set compared to controls. These VUS in desmosomal genes seem to be relevant with a significant enrichment in cases compared to controls according to our burden tests. This represents about 15% of ACR patients in this study for whom molecular diagnosis may be inconclusive due to the miss classification in VUS. We showed that the degree of conservation, pathogenicity, and position in the gene and protein might represent clues to discriminate PV among the VUS. Furthermore, caution has to be taken especially regarding rare variants found in large genes such as *RYR2* containing the highest number of VUS likely due to its length. This gene is today refuted by the ACR expert group in ClinGen (James et al., 2021). Aside from molecular functional studies, recruitment of relatives and the study of the correlation between genotypes and phenotypes among large pedigrees remains the most informative way to assess the pathogenicity of a variant. Indeed, ACR loss-of-function variants should be interpreted in relation to an established phenotype of ACR since some have also been found in the general population (Carruth Eric et al., 2019).

Among the other ACR-associated genes (*JUP*, *TMEM43*, *LMNA*, *PLN*, *RYR2*, *SCN5A*, and *TGFβ3*) no enrichment in PV or LPV or in VUS was identified. Even if specific variants in such genes have been associated with ACRR, mostly in the context of founder mutation studies (Merner et al., 2008; Zwaag et al., 2012), our study indicates that (1) the prevalence of variants among these genes is low or absent in our patient set, (2) an equivalent number of rare variants are found in cases and controls leading to a low molecular diagnostic yield and difficulty to interpret variants identified in such genes, respectively. Sixty other genes present on the panel and corresponding to genes associated with inherited arrhythmia and cardiomyopathies do not present PVs nor any enrichment in PV or LPV or in VUS, suggesting a very likely modest implication in the ACR pathophysiology. This also indicates that the missing heritability for ACR may reside in other genes or in other variants not investigated in this study, such as large rearrangements, upstream variants (Christensen et al., 2019), or a combination of common variants.

According to the recent guidelines of the Heart Rythm Society (Towbin et al., 2019) (published after our gene panel selection), the molecular diagnosis should be based on 14 genes. Among the 10 genes present in our panel, only desmosomal genes seem to be enriched in likely causal variants. These desmosomal genes are part of the actionable genes (Kalia et al., 2017) and PV, LPV, and VUS should be considered for the diagnosis of cardiomyopathy and clinical management and also for family screening. Moreover, *LDB3* and *NKX2.5*, which are recommended to be screened, count 15 and 12 VUS in controls, respectively, and none in the ACR patient set. Furthermore, a study by Costa et al. (2021) on 79 ACR patients showed that about 10% of patients had lost their definitive diagnosis of ACR due to a reclassification of variants mostly in non-*PKP2* desmosomal genes. Recently, Ye et al. (2019) showed about 10% of variants previously associated with ACR were found unlikely to be

associated with this pathology, particularly in nondesmosomal genes. Our study confirms an effective molecular diagnosis yield by screening as a first intent the desmosomal genes.

The guidelines proposed by the ACMG were used in this study to standardize the assessment of pathogenicity of variants using two tools: Intervar and eVAI. According to the bioinformatic tools, the classification may change and the limits and characteristics of the tool used must be known. In our study, 30% of variants was annotated pathogenic/likely pathogenic with eVAI and uncertain significance with InterVar (19/63 variants including 6 missense, 10 frameshift and 3 nonsense variants). This difference in classification underlies the caution required in the interpretation of variants and remains to be critical for variant interpretation, especially knowing that a PV represents a major criterion in the Task Force classification. Fortunately, algorithms and tools are improving and may provide a strong support in molecular diagnosis (Walsh et al., 2021).

In 10 cases, two rare variants were identified but no correlation with the phenotype severity could be established. Even if the correlation between the phenotype of patients and the genotype is challenging, due to the high variability of clinical presentation, genetic heterogeneity, and interpretation of variants, our study supports previous studies with the predominance of genes according to the type of ventricular damage (e.g., *DSP* for left ventricular damage) (Sakamoto et al., 2019; Towbin et al., 2019). We observed an earlier onset in patients carrying a variant compared to those negative for the gene panel, strengthening the required step to lead a comprehensive clinical investigation in all the relatives, even in the young.

This study evaluated for the first time the burden of rare variants within cardiomyopathy and arrhythmia susceptibility genes in a large ACR patient set. We showed that only desmosomal genes (*PKP2*, *DSP*, *DSG2*, and *DSC2*) present a significant burden of (likely) PVs among cases tested. We also demonstrated that VUS in desmosomal genes might account to explain 15% of ACR cases and should then be considered for molecular diagnosis of ACR. Among the other genes, no evidence of enrichment was detected, suggesting an extreme caution in the interpretation of these genetic variations in a molecular diagnostic context when not supported by functional or segregation data. PVs seem to be associated with the severity of the disease and an earlier onset, confirming the importance to carry out a presymptomatic diagnosis in relatives. A predominance of left ventricular dysfunction in *DSP* variant carriers and higher rhythmic events in *PKP2* carriers should be considered in the clinical management of the patient.

ACKNOWLEDGMENT

The authors are greatly indebted to the patients included in the study. They thank Marie-France Le Cunff and Noémie Bourgeais for assistance in patient recruitment. The authors gratefully thank all members of the FranceGenRef consortium supported by Grant No. ANR-10-LABX-0013. J. B. is supported by the research program Etoiles montantes des Pays de la Loire REGIOCARD RPH081-U1087-REG-PDL and this study is part of the CRITERIA project funded by the French Federation of Cardiology. We are most grateful to the Genomics and Bioinformatics Core Facility of Nantes (GenoBiRD, Biogenouest, IFB) for its technical support. We thank the biological resource centre for biobanking (CHU Nantes, Nantes Université, Centre de ressources biologiques (BB-0033-00040), Nantes, France) (Bravo et al., 2015). Richard Redon was supported by the Prix des Journées de l'Avenir 2019 funded by la Fondation de l'Avenir et la Ligue Nationale de Basket.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Goudal, A., Karakachoff, M., Lindenbaum, P., Baron, E., Bonnaud, S., Kyndt, F., Arnaud, M., Minois, D., Bourcereau, E., Thollet, A., Deleuze, J.-F., Genin, E., Wiart, F., Pasquié, J.-L., Galand, V., Sacher, F., Dina, C., Redon, R., Bezieau, S., … Barc, J. (2022). Burden of rare variants in arrhythmogenic cardiomyopathy with right dominant form-associated genes provides new insights for molecular diagnosis and clinical management. *Human Mutation*, 43, 1333–1342. https://doi.org/10.1002/humu.24436