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Research paper

Reanalysis and reclassification of rare genetic variants associated with inherited arrhythmogenic syndromes

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ABSTRACT

Background: Accurate interpretation of rare genetic variants is a challenge for clinical translation. Updates in recommendations for rare variant classification require the reanalysis and reclassification. We aim to perform an exhaustive re-analysis of rare variants associated with inherited arrhythmogenic syndromes, which were classified ten years ago, to determine whether their classification aligns with current standards and research findings.
Methods: In 2010, the rare variants identified through genetic analysis were classified following recommendations available at that time. Nowadays, the same variants have been reclassified following current American College of Medical Genetics and Genomics recommendations.
Findings: Our cohort included 104 cases diagnosed with inherited arrhythmogenic syndromes and 17 postmortem cases in which inherited arrhythmogenic syndromes was cause of death. 71.87% of variants change their classification. While 65.62% of variants were classified as likely pathogenic in 2010, after reanalysis, only 17.96% remain as likely pathogenic. In 2010, 18.75% of variants were classified as uncertain role but nowadays 60.15% of variants are classified of unknown significance.
Interpretation: Reclassification occurred in more than 70% of rare variants associated with inherited arrhythmogenic syndromes. Our results support the periodical reclassification and personalized clinical translation of rare variants to improve diagnosis and adjust treatment.
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1. Introduction

Advances in gene sequencing technology have made genetic testing in clinical diagnosis more accessible by increasing the number of analysed genes, decreasing costs, and reducing the amount of time required for analysis [1]. For an adequate translation of genetic data to clinical practice, and in order to manage the inherited conditions, it is critical to perform an appropriate interpretation of the genetic variant [2]. Sudden death may be the first manifestation of

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Research in context

Evidence before this study

The American College of Medical Genetics and Genomics (ACMG) current recommendations include guidelines for obtaining an accurate assessment of rare variants. However, there is a lack of available data for each rare variant, and many remain of uncertain significance. Therefore, many of the families affected with inherited arrhythmic syndrome remain with an inconclusive genetic diagnosis, which is not helpful in clinical decision-making. Accurate interpretation of rare genetic variants is a challenge for clinical translation. Updates in recommendations for rare variant classification require the reanalysis and reclassification. An exhaustive review of the literature concerning each variant was performed through December 2019. Data was collected from: HGMD (www.hgmd.org), ClinVar (www.ncbi.nlm.nih.gov/clinvar/intro/), the National Center for Biotechnology Information SNP database (www.ncbi.nlm.nih.gov/SNP), Index Copernicus (en.indexcopernicus.com), Google Scholar (scholar.google.es), Springer Link (link.springer.com), Science Direct (www.sciencedirect.com), Excerpta Medica Database (www.elsevier.com/solutions/embase-biomedical-research), and the IEEE Xplore Digital Library (ieeexplore.ieee.org/Xplore/home.jsp). All genetic variants included in our study were compared to data from Exome Variant Server (EVS; evs.gs.washington.edu/EVS), and Genome Aggregation Database (gnomAD gnomad.broadinstitute.org/).

Added value of this study

Establishing a definite pathogenicity of rare genetic variants helps for clinical diagnosis of inherited arrhythmogenic syndromes but also helps to adopt therapeutic measures for prevention of sudden death. We have performed an accurate genetic reinterpretation of variants classified 10 years ago. Reclassification occurred in more than 70% of rare variants associated with inherited arrhythmogenic syndromes. These changes may influence clinical decisions adopted 10 years ago.

Implications of all the available evidence

Currently, classification of a genetic variant follows guidelines published in 2015 by ACMG/AMP. These recommendations are based on available data concerning the variant at the moment of classification. Data available 10 years ago is not the same as now. Therefore, reclassification and reinterpretation of a variant should be updated periodically to improve diagnosis and adjust treatment despite no concrete timeframe for this being established. In the light of our results, we propose that rare variants associated with inherited arrhythmogenic syndromes should be reanalysed within five years if already classified following ACMG recommendations, since this seems to be adequate to manage the rapid obsolescence of genetic data interpretations. In addition, our results support further urgent reanalysis of each IAS rare variant if they were not classified originally following ACMG recommendations.

The American College of Medical Genetics and Genomics (ACMG) current recommendations include guidelines for obtaining an accurate assessment of rare variants [4]. However, there is a lack of available data for each rare variant, and many remain of uncertain significance. Therefore, many of the families affected with IAS remain with an inconclusive genetic diagnosis, which is not helpful in clinical decision-making [5]. Rare variants classified as inconclusive are disregarded, and only clinical and family history are referenced in determining risk-assessment and clinical management [4].

Clinical and functional data on rare IAS variants published in the last ten years has helped clarify their roles and improved their classification. Continuous reclassification is recommended to update their roles before clinical translation. Such re-evaluation may serve to improve psychological outcomes and risk stratification while promoting personalized management [6, 7]. Only a few reports have addressed this idea in recent years [8–10]. In the present study, we describe the reclassification of rare IAS variants reported ten years ago by our laboratory to update their roles following current guidelines.

2. Materials and methods

2.1. Samples

This retrospective study reanalysed rare IAS variants classified in our laboratory ten years ago (during the year 2010). Rare variants were originally classified following recommendations available in 2010 as pathogenic (P), likely pathogenic (LP), variant of unknown significance (VUS), or as likely benign (LB) [11]. Variants classified as Benign in 2010 were not reanalysed due to global frequencies higher than 1%, and already identified ten years ago as common variants. All rare variants were identified in two groups: samples from patients with a definite clinical diagnosis of IAS or post-mortem samples without a conclusive cause of death but with suspected IAS. Genetic analysis was approved by the ethics committee of Hospital Josep Trueta (Girona, Spain) following the Helsinki II declaration. Both clinical and genetic data concerning all patients were kept confidential. Written informed consent was obtained from all patients included in the study. In post-mortem cases, a family member authorized the study or judge/legal authority included molecular autopsy as part of legal process.

2.2. Genetic analysis

Genomic DNA was extracted with Chemagic MSM I from whole blood (Chemagic human blood) or saliva (Chemagic Oragene Saliva). Concentration was determined along with purity using a NanoDrop1000 spectrophotometer (Thermo scientific). Genomic DNA was amplified by polymerase chain reaction (PCR) using intronic primers for each exon of all the genes analysed. The PCR product was purified by ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and directly sequenced by dideoxy chain-termination method in an ABI Prism Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing was processed in a 3130XL Genetic Analyzer (Applied Biosystems) and analysed by SeqScape Software v2.5 (Life Technologies).

The genes associated with each IAS subtype were analysed following the prevailing recommendations in 2010 [11]. Genetic analysis for arrhythmogenic cardiomyopathy (ACM) included *PKP2, DSP, DSC2, DSG2, DES, JUP,* and *TGFB3*; analysis for Brugada syndrome (BrS) included *SCN5A*; analysis for catecholaminergic polymorphic ventricular tachycardia (CPVT) included *RYR2* and *CASQ2*; analysis for dilated cardiomyopathy (DCM) included *LMNA*; analysis for hypertrophic cardiomyopathy (HCM) included *MYH7, MYBPC3, TNNT2,* and *TNNI3;* analysis for long QT Syndrome (LQTS) included *KCNQ1, KCNH2,* and *SCN5A;* and finally, analysis for sick sinus syndrome (SSS) included *SCN5A* and *HCN4*. The genes analysed in post-mortem cases were *SCN5A, KCNQ1, KCNH2, KCNE1, KCNE2, KCNE3, RYR2,* and *CASQ2*. All

an inherited arrhythmic syndrome (IAS), thus early identification with genetic technology may help adopt preventive measures and reduce the risk of lethal episodes in family members. Genetic analysis may also be determinant in identifying causality in sudden death deemed inconclusive after a comprehensive autopsy. For these reasons current guidelines recommend genetic analysis in diagnosed patients and relatives who may be at risk, despite remaining asymptomatic [3].

original sequences obtained in 2010 were comprehensively reanalysed with updated software (SeqScape v2.7, Applied Biosystems) to identify any alteration not identified at the time of report.

2.3. Data

An exhaustive review of the literature concerning each variant was performed through December 2019. Data was collected from: HGMD (www.hgmd.org), ClinVar (www.ncbi.nlm.nih.gov/clinvar/intro/), the National Center for Biotechnology Information SNP database (www.ncbi.nlm.nih.gov/SNP), Index Copernicus (en.indexcopernicus.com), Google Scholar (scholar.google.es), Springer Link (link.springer.com), Science Direct (www.sciencedirect.com), Excerpta Medica Database (www.elsevier.com/solutions/embase-biomedical-research), and the IEEE Xplore Digital Library (ieeexplore.ieee.org/Xplore/home.jsp). All genetic variants included in our study were compared to data from Exome Variant Server (EVS; evs.gs.washington.edu/EVS), and Genome Aggregation Database (gnomAD gnomad.broadinstitute.org/).

2.4. Classification

Ten years ago, variants were classified following an in-house algorithm including multiple parameters such as population frequencies, *in silico* predictions, and published functional data. Algorithms followed recommendations available in 2010 [11]. Part of this in-house algorithm focused on inherited arrhythmogenic disorders was published in 2015 [12]. Nowadays, all variants have been reclassified according to ACMG standards and guidelines for the interpretation of sequence variants as P, LP, VUS, LB, or benign (B) [4]. The PM2 item in the ACMG classification was considered fulfilled if Minor Allele Frequency (MAF) in relevant population databases was ≤0.1% [13]. The vast majority of reported pathogenic variants in IAS are extremely rare (<0.01%) [14]. High degree of pathogenicity (item PVS1) should only be used for rare variants in genes where loss of function is a well-established disease mechanism [15]. Genetic data were independently evaluated and classified by three clinical genetic experts. All investigators discussed and agreed on a final classification of all variants to avoid bias.

3. Results

3.1. Cohort

Our retrospective study included 121 cases, all Caucasian, with an even gender distribution (65 men; 53.71% and 56 women; 46.28%). Ages ranged from 19 to 51 years of age (mean age: 39.2 years). The first cohort (named "clinical group" −CL-) included 104 cases (85.95%) with a definite clinical diagnosis of IAS. Definitively, CL included 48 cases of BrS, 34 cases of ACM, 10 cases of LQTS, 7 cases of HCM, 4 cases of CPVT, and only one case diagnosed with DCM (Fig. 1). Suspicious cases with an inconclusive diagnosis were not included to avoid bias in the reclassification of genetic variants. The second cohort (named "post-mortem group" −PM-) comprised 17 post-mortem samples (14.05%) with IAS as the likely cause of death (Fig. 1). Exhaustive complete autopsies were performed in all cases, including toxicological analysis, before final judgment. Review of clinical and forensic data did not change the diagnostic decisions from ten years ago.

3.2. Genetics

One hundred twenty-eight rare variants were localized in 17 genes: 55 in *SCN5A*, 17 in *PKP2*, 9 in *KCNH2* and *RyR2*, 8 in *DSG2*, 6 in *KCNQ1*, 4 in *MYBPC3, MYH7* and *DSP*, 3 in *JUP*, 2 in *DSC2* and *KCNE2*, and 1 in *KCNE3, DES, LMNA* and *TGFB3*. All identified variants were exonic except one intronic variant (c.3840+1G>A_*SCN5A*, case 28). Of the 127 exonic variants, 96 were missense and 31 were radical (ten

nonsense, fifteen deletions and six insertions). All cases carried at least one rare variant except for seven cases that carried two rare variants (case 26, LQTS; case 28, BrS; case 65, BrS; case 72, LQTS; case 79, ACM; case 107, ACM; and case 113, HCM). Original classification concluded that there were 19 P variants (14.84%), 84 LP variants (65.62%), 24 VUS variants (18.75%), and only one LB variant (0.78%) (Fig. 1) (Tables 1−3).

Reanalysis following current ACMG guidelines conferred significant changes in 71.87% (92 of 128) of the rare variants. One variant changed from LB to VUS (0.7%), 6 rare variants changed from VUS to LB (4.6%), and one changed from VUS to LP (0.7%). Four rare variants initially classified as LP in 2010 were changed to LB (3.1%), 59 were changed to VUS (46.09%), and 10 were changed to P (7.8%). Ten rare variants classified as P in 2010 were downgraded to LP in the reclassification (7.8%). In 2010, we classified 24 rare variants as VUS (18.75%) and after reanalysis, there were 77 classified as VUS (60.15%; 17 rare variants had no modification and 60 were reclassified). Originally, the majority were LP variants (84 of 128; 65.62%), but after reanalysis, the predominant classification of the variants was VUS (77 of 128; 60.65%) in contrast to 18.75% (24 of 128), ten years ago. Just twenty-three of 128 (17.96%) were classified as LP. After reanalysis, VUS became the predominant group (77 of 128; 60.65%) in contrast to 18.75% (24 of 128) ten years ago. In 2010, only one variant (0.78%) was classified as LB while after the reanalysis, 7.8% (10 of 128) were classified LB. The percentage of P variants was similar in both classifications (19 of 128; 14.84% in 2010 and 18 of 128; 14.06% after the reanalysis) (Fig. 2).

A total of 128 rare variants were analysed from 121 cases of IAS (CL group). In 88 cases (82.72%), the variant classification suffered a modification according to the current ACMG recommendations: 72 of the 104 cases with an IAS diagnosis (69.23%) and 16 of the 17 (94.11%) cases from the post-mortem cohort. Originally, in the post-mortem cohort, 82.23% (14 of 17) of variants were classified as LP, while after the reanalysis the same percentage was classified as VUS (14/17; 82.82%). In the IAS cohort, 63.06% (70 of 111) of the variants were initially classified as LP. However, after the reanalysis, 56.75% (63 of 111) were classified as VUS. Differences on the classification were observed also between missense and radical variants. In the 2010 classification, 49 of the 79 missense variants (62.02%) were classified as LP, while the major classification after the reanalysis was VUS (62 of 70; 78.48%). Radical variants were predominantly classified as LP in 2010 (21 of 31; 67.74%); although after the reanalysis a 58.06% of the radical variants were classified as P. The rare variants located in five genes (*DES, JUP, KCNE3, LMNA,* and *MYH7*) did not suffer any difference in final classification (Fig. 1) (Tables 3 and 4).

In 48 cases of BrS, most rare variants were originally classified as LP (41 of 49; 83.67%) but after reanalysis, most were classified as VUS (34 of 49; 69.38%). In case number 28, diagnosed with BrS, an intronic variant was originally classified as P but after reanalysis, was classified as LP. In ten cases diagnosed with LQTS, 50% (6 of 12 cases) were classified as P but almost all rare variants have been reclassified as LP (8 of 12; 66.66%). In four cases diagnosed with CPVT, all rare variants (4 of 4; 100%) were originally classified as LP but after reanalysis, the same four rare variants were classified as VUS. A total of 34 cases diagnosed with ACM were originally classified as LP (52.77%; 19 of 36), but after reanalysis, most were classified as VUS (16 of 36; 44.44%). In the one case diagnosed with DCM, the rare variant originally classified as VUS remained at the same level of pathogenicity. In seven cases diagnosed with HCM, 50% of rare variants were originally classified as VUS (4 of 8) and after reanalysis, the same percentage of VUS was maintained (Fig. 1) (Tables 1, 2 and 4).

4. Discussion

Genetic testing in patients diagnosed with IAS is highly recommended both in clinical and medico-legal settings, since death is
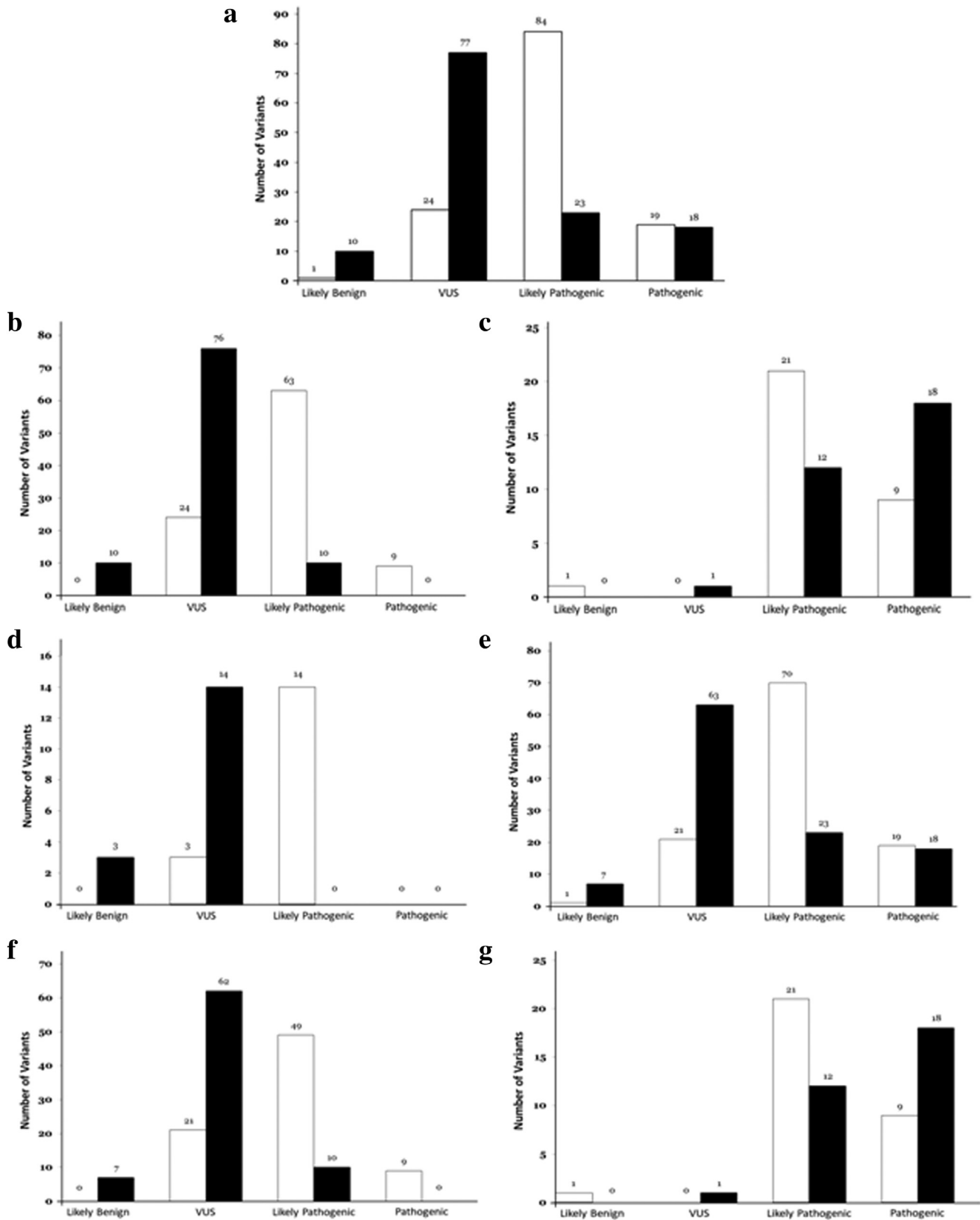
Fig. 1. Distribution of rare variants. White columns represent the original classification (2010). Black columns represent the classification after the reanalysis. (a) Global classification of rare variants. (b) Global classification of rare missense variants. (c) Global classification of rare radical variants. (d) Classification of rare variants in Post-Mortem group. (e) Classification of rare variants in Clinical group. (f) Classification of rare missense variants in Clinical group. (g) Classification of rare radical variants in Clinical group.

often the first manifestation of disease [3]. However, misinterpretation of rare variant designations may lead to inaccurate genetic diagnoses and/or the adoption of unnecessary and/or inappropriate

therapeutic approaches resulting in an increased morbidity and mortality. Therefore, one of the main current challenges in genetic analysis is determining the pathogenic role of rare variants. Identifying a

Table 1
Variant genetic data.

Index case	Disease	Gene	Nucleotide	Protein	dbSNP	gnomAD	HGMD (disease)	ClinVar	2010 classification	2020 classification (ACMG)
1	BrS	SCN5A	c.5464_5467delTCTG	p.(Glu1823Hisfs*10)	rs794728924	1/249656 (0.0004%)	CD077699 (SSS)	P	P	P
2	ACM	PKP2	c.1613G>A	p.(Trp538*)	rs193922672	4/251382 (0.001%)	CM061177 (ACM)	LP	LP	P
3	BrS	SCN5A	c.1717C>T	p.(Gln573*)	NA	NA	CM100660 (BrS)	NA	LP	P
4	BrS	SCN5A	c.4477A>T	p.(Lys1493*)	NA	NA	CM100735 (BrS)	NA	LP	P
5	BrS	SCN5A	c.2865_2866delGA	p.(Glu955Aspfs*74)	rs756159737	4/248468 (0.001%)	NA	NA	LP	LP
6	BrS	SCN5A	c.1721delG	p.(Gly574Aspfs*49)	NA	NA	CD100781 (BrS)	NA	LP	LP
7	ACM	DSG2	c.146G>A	p.(Arg49His)	rs121913006	1/249482 (0.0004%)	CM061700 (ACM)	LP	VUS	VUS
8	LQTS	KCNQ1	c.421G>A	p.(Val141Met)	rs199472687	NA	CM056972 (AF)	LP	P	LP
9	BrS	SCN5A	c.4562T>A	p.(Ile1521Lys)	rs199473617	NA	CM100736 (BrS)	NA	LP	VUS
10	BrS	SCN5A	c.4534C>T	p.(Arg1512Trp)	rs137854602	14/251272 (0.005%)	CM994138 (BrS)	VUS	LP	VUS
11	BrS	SCN5A	c.5272_5274delATC	p.(Ile1758del)	NA	NA	CD1810427 (PCCD)	NA	LB	VUS
12	BrS	SCN5A	c.707T>G	p.(Leu236Arg)	NA	NA	.	NA	LP	VUS
13	ACM	DSG2	c.1381C>T	p.(Gln461*)	rs1212557775	NA	CM1314709 (ACM)	NA	LP	LP
14	BrS	SCN5A	c.4978A>G	p.(Ile1660Val)	rs199473625	8/251490 (0.003%)	CM057204 (LQTS)	VUS	LP	VUS
15	BrS	SCN5A	c.2893C>T	p.(Arg965Cys)	rs199473180	16/246378 (0.006%)	CM024644 (BrS)	VUS	LP	VUS
16	ACM	JUP	c.1028G>A	p.(Ser343Asn)	NA	NA	NA	NA	VUS	VUS
17	BrS	SCN5A	c.4352T>C	p.(Val1451Ala)	NA	NA	NA	NA	LP	VUS
18	ACM	JUP	c.475G>T	p.(Val159Leu)	rs782702266	11/269700 (0.004%)	CM1010258 (ACM)	VUS	VUS	VUS
19	BrS	SCN5A	c.4493T>C	p.(Met1498Thr)	rs199473263	NA	CM057203 (LQTS)	VUS	LP	VUS
20	ACM	DSP	c.2956C>T	p.(Gln986*)	NA	NA	CM1310184 (ACM)	NA	LP	P
21	ACM	PKP2	c.2013delC	p.Lys672Argfs*12	rs764817683	2/251350 (0.0007%)	CD061457 (ACM)	P	P	P
22	BrS	SCN5A	c.2550_2551dupGT	p.Phe851Cysfs*19	rs397514450	NA	CI055774 (DCM)	P	P	P
23	BrS	SCN5A	c.4856delC	p.Pro1619Argfs*12	NA	NA	CD100798 (BrS)	NA	LP	P
24	BrS	SCN5A	c.1936del	p.Gln646Argfs*5	rs727505158	1/31374 (0.003%)	CD100782 (BrS)	P	LP	P
25	BrS	SCN5A	c.5174C>T	p.(Pro1725Leu)	rs199473301	5/251170 (0.001%)	CM097849 (LQTS)	VUS	LP	VUS
26	LQTS	KCNH2	c.2639G>T	p.(Gly880Val)	NA	NA	CM150041 (LQTS)	NA	P	LP
		KCNH2	c.1838C>T	p.(Thr613Met)	rs199473524	NA	CM990761 (LQTS)	P	LP	VUS
27	ACM	PKP2	c.2203C>T	p.(Arg735*)	rs121434421	1/251356 (0.0003%)	CM043061 (ACM)	P	P	P
28	BrS	SCN5A	c.3840+1G>A	NA	NA	NA	CS099837 (BrS)	NA	P	LP
		SCN5A	c.5068G>A	p.(Asp1690Asn)	rs1060499900	1/251488 (0.0003%)	CM136071 (BrS)	VUS	LP	VUS
29	BrS	SCN5A	c.2669T>C	p.(Ile890Thr)	NA	NA	CM130365 (BrS)	NA	LP	VUS
30	BrS	SCN5A	c.1705dupC	p.(Arg569Profs*152)	NA	NA	CI1510495 (BrS)	NA	LP	LP
31	BrS	SCN5A	c.1872dupA	p.(Glu625Argfs*96)	NA	NA	CI1510496 (BrS)	NA	LP	LP
32	ACM	PKP2	c.1912C>T	p.(Gln638*)	rs397517012	1/251302 (0.0003%)	CM043056 (ACM)	P	P	P
33	BrS	SCN5A	c.2729C>T	p.(Ser910Leu)	rs199473175	1/250430 (0.0003%)	CM024643 (BrS)	LP	LP	VUS
34	ACM	PKP2	c.604dupG	p.(Val202Glyfs*14)	NA	NA	CI146422 (ACM)	NA	LP	LP
35	ACM	DSG2	c.137G>A	p.(Arg46Gln)	rs121913008	1/280866 (0.00003%)	CM061701 (ACM)	LP	LP	VUS
36	BrS	SCN5A	c.2962C>T	p.(Arg988Trp)	rs768691853	5/238498 (0.002%)	CM137981 (BrS)	VUS	LP	VUS
37	LQTS	SCN5A	c.5859_5862delTGAG	p.(Ser1953Argfs*84)	rs758317466	1/246198 (0.0004%)	NA	NA	LP	LP
38	BrS	SCN5A	c.4213G>A	p.(Val1405Met)	rs199473239	NA	CM100715 (BrS)	VUS	P	LP
39	BrS	SCN5A	c.361C>T	p.(Arg121Trp)	rs199473556	NA	CM095355 (BrS)	VUS	LP	VUS
40	BrS	SCN5A	c.1100G>A	p.(Arg367His)	rs28937318	NA	CM020301 (SUNDS)	LP	P	LP
41	BrS	SCN5A	c.5177C>G	p.(Pro1726Arg)	NA	NA	NA	NA	LP	VUS
42	ACM	TGFB3	c.1230A>C	p.(Lys410Asn)	NA	NA	NA	NA	LP	VUS
43	ACM	DSG2	c.2440T>C	p.(Cys814Arg)	NA	NA	CM146425 (ACM)	NA	LP	VUS
44	ACM	PKP2	c.275T>A	p.(Leu92*)	rs763639737	2/251424 (0.0007%)	CM102825 (ACM)	P	P	P
45	ACM	DES	c.407T>A	p.(Leu136His)	rs397516695	15/213206 (0.007%)	CM159728 (DCM)	VUS	VUS	VUS
46	ACM	DSP	c.6208G>A	p.(Asp2070Asn)	rs41302885	1118/282114 (0.39%)	CM198079 (BrS)	LB	VUS	LB
47	LQTS	KCNQ1	c.898G>A	p.(Ala300Thr)	rs120074187	12/249914 (0.004%)	CM983511 (LQTS)	VUS	P	LP
48	BrS	SCN5A	c.2548G>A	p.(Val850Met)	rs911293694	2/251416 (0.0007%)	NA	NA	LP	VUS
49	BrS	SCN5A	c.5380T>A	p.(Phe1794Ile)	NA	NA	NA	NA	LP	VUS
50	BrS	SCN5A	c.4018G>A	p.(Val1340Ile)	rs199473605	13/ 282822 (0.004%)	CM100703 (BrS)	VUS	LP	VUS
51	HCM	MYH7	c.5779A>T	p.(Ile1927Phe)	rs767300277	11/251320 (0.004%)	CM082963 (HCM)	VUS	VUS	VUS
52	BrS	SCN5A	c.2168dupT	p.(Thr724Hisfs*21)	NA	NA	NA	NA	LP	LP

Table 1 (*Continued*)

Index case	Disease	Gene	Nucleotide	Protein	dbSNP	gnomAD	HGMD (disease)	ClinVar	2010 classification	2020 classification (ACMG)
53	BrS	SCN5A	c.2314G>A	p.(Asp772Asn)	rs199473157	5/249248 (0.002%)	CM097652 (LQTS)	VUS	LP	VUS
54	BrS	SCN5A	c.4219dupG	p.(Ala1407Glyfs*13)	NA	NA	NA	NA	LP	P
55	ACM	DSG2	c.473T>G	p.(Val158Gly)	rs191143292	1537/280564 (0.54%)	CM100657 (ACM)	LB	VUS	LB
56	BrS	SCN5A	c.1577G>A	p.(Arg526His)	rs45627438	14/242632 (0.005%)	CM100657 (BrS)	VUS	LP	VUS
57	BrS	SCN5A	c.1120T>C	p.(Trp374Arg)	NA	NA	NA	NA	LP	VUS
58	LQTS	KCNQ1	c.1016T>C	p.(Phe339Ser)	rs199472759	NA	CM073160 (LQTS)	LP	P	LP
59	ACM	PKP2	c.1162C>T	p.(Arg388Trp)	rs766209297	1/251320 (0.0003%)	CM097906 (ACM)	LP	LP	VUS
60	BrS	SCN5A	c.4345T>C	p.(Tyr1449His)	NA	NA	NA	NA	VUS	VUS
61	BrS	SCN5A	c.481G>A	p.(Glu161Lys)	rs199473062	1/240992 (0.0004%)	CM023671 (BrS)	LP	VUS	VUS
62	ACM	DSG2	c.166G>A	p.(Val56Met)	rs121913013	518/280886 (0.18%)	CM070918 (ACM)	LB	VUS	LB
63	ACM	PKP2	c.1378G>A	p.(Asp460Asn)	rs794729106	NA	CM1213407 (ACM)	NA	P	LP
64	ACM	DSP	c.6361G>C	p.(Gly2121Arg)	rs368227724	1/251360 (0.0003%)	NA	NA	LP	VUS
65	BrS	SCN5A	c.1579G>C	p.(Gly527Arg)	rs763550164	8/243942 (0.003%)	NA	VUS	LP	VUS
		SCN5A	c.3929C>G	p.(Pro1310Arg)	NA	NA	NA	NA	LP	VUS
66	BrS	SCN5A	c.2236G>A	p.(Glu746Lys)	rs199473582	5/248406 (0.002%)	CM100669 (BrS)	VUS	LP	VUS
67	CPVT	RYR2	c.14639T>C	p.(Val4880Ala)	rs1242723821	NA	HM030023 (CPVT)	NA	LP	VUS
68	ACM	PKP2	c.2576delA	p.(Lys859Argfs*72)	NA	NA	CD146431 (ACM)	NA	LP	LP
69	ACM	PKP2	c.1643delG	p.(Gly548Valfs*15)	rs794729137	NA	CD043194 (ACM)	P	P	P
70	BrS	SCN5A	c.2633G>A	p.(Arg878His)	rs199473587	NA	CM100676 (BrS)	NA	LP	VUS
71	ACM	PKP2	c.2060T>G	p.(Leu687Arg)	rs794729113	NA	NA	VUS	VUS	VUS
72	LQTS	KCNH2	c.712G>C	p.(Gly238Arg)	NA	NA	NA	NA	VUS	VUS
		KCNQ1	c.944A>G	p.(Tyr315Cys)	rs74462309	NA	CM981127 (LQTS)	LP	VUS	LP
73	BrS	SCN5A	c.5859_5862delTGAG	p.(Ser1953Argfs*84)	rs758317466	1/246198 (0.0004%)	NA	NA	LP	LP
74	ACM	PKP2	c.1759G>A	p.(Val587Ile)	rs146102241	616/251180 (0.24%)	NA	LB	VUS	LB
75	BrS	SCN5A	c.4981G>A	p.(Gly1661Arg)	NA	NA	CM100750 (BrS)	NA	LP	VUS
76	ACM	DSG2	c.527C>T	p.(Thr176Ile)	rs536617217	4/280698 (0.001%)	NA	VUS	LP	VUS
77	LQTS	KCNH2	c.1744C>T	p.(Arg582Cys)	rs121912508	NA	CM990759 (LQTS)	P	P	LP
78	BrS	SCN5A	c.5177C>A	p.(Pro1726His)	NA	NA	NA	NA	LP	VUS
79	ACM	DSC2	c.835C>T	p.(Arg279Cys)	rs193922708	12/251360 (0.004%)	CM146543 (ACM)	VUS	LP	LP
		PKP2	c.1882delC	p.(Gln628Argfs*28)	NA	NA	CD146544 (ACM)	NA	LP	VUS
80	BrS	SCN5A	c.4052T>G	p.(Met1351Arg)	rs199473232	NA	CM100707 (BrS)	NA	LP	VUS
81	BrS	SCN5A	c.5092G>A	p.(Ala1698Thr)	rs199473295	3/251490 (0.001%)	CM100753 (BrS)	VUS	VUS	VUS
82	SCD	RYR2	c.2047G>A	p.(Glu683Lys)	NA	NA	NA	NA	LP	VUS
83	SCD	RYR2	c.12056T>C	p.(Met4019Thr)	rs886039150	NA	CM173280 (MI)	VUS	LP	VUS
84	SCD	KCNE2	c.29C>T	p.(Thr10Met)	rs199473648	66/282722 (0.023%)	CM055291 (LQTS)	VUS	VUS	LB
85	SCD	KCNH2	c.2674C>T	p.(Arg892Cys)	rs201627778	111/277590 (0.039%)	CM1413446 (SCD)	VUS	LP	VUS
86	SCD	RYR2	c.12919C>T	p.(Arg4307Cys)	rs200092869	86/248746 (0.03%)	NA	VUS	LP	VUS
87	SCD	KCNE1	c.253G>A	p.(Asp85Asn)	rs1805128	2637/282814 (0.9%)	CM040436 (LQTS, DI)	B	LP	LB
88	BrS	SCN5A	c.3911C>T	p.(Thr1304Met)	rs199473603	46/279030 (0.01%)	CM992663 (LQTS)	VUS	LP	VUS
89	SCD	SCN5A	c.1440A>C	p.(Lys480Asn)	rs752966781	2/249180 (0.0008%)	NA	VUS	LP	VUS
90	CPVT	RYR2	c.14667C>G	p.(Phe4889Leu)	NA	NA	NA	NA	LP	VUS
91	HCM	MYBPC3	c.2827C>T	p.(Arg943*)	rs387907267	3/247124 (0.001%)	CM032959 (HCM)	P	LP	P
92	SCD	RYR2	c.8145G>T	p.(Glu2715Asp)	rs200420897	14/134624 (0.01%)	NA	VUS	LP	VUS
93	SCD	KCNH2	c.865G>A	p.(Glu289Lys)	rs199472880	7/35014 (0.01%)	CM097827 (LQTS)	VUS	LP	VUS
94	SCD	KCNH2	c.2860C>T	p.(Arg954Cys)	rs141401803	8/217960 (0.003%)	CM070176 (SIDS)	VUS	LP	VUS
95	SCD	SCN5A	c.3530C>G	p.(Pro1177Arg)	NA	NA	NA	NA	LP	VUS
96	LQTS	KCNQ1	c.1097G>A	p.(Arg366Gln)	rs199473410	1/251240 (0.0003%)	CM002330 (LQTS)	P	P	LP
97	SCD	SCN5A	c.5054_5055delinsTT	p.(Glu1685Val)	NA	NA	NA	NA	LP	VUS
98	ACM	JUP	c.2069A>G	p.(Asn690Ser)	rs147628503	29/282402 (0.01%)	CM1416877 (Autism)	VUS	VUS	VUS
99	HCM	MYBPC3	c.3328delA	p.(Met1110Trpfs*79)	NA	NA	CD1710421 (HCM)	NA	LP	P
100	SCD	KCNH2	c.2941A>G	p.(Ser981Gly)	rs76649554	75/276264 (0.027%)	NA	VUS	LP	VUS
101	HCM	MYH7	c.4377G>C	p.(Lys1459Asn)	rs201307101	NA	CM042424 (HCM)	NA	VUS	VUS
102	CPVT	RYR2	c.217C>G	p.(Leu73Val)	rs777753947	1/249224 (0.0004%)	CM1413452 (SCD)	NA	LP	VUS
103	LQTS	KCNQ1	c.1861G>A	p.(Gly621Ser)	rs199472820	9/177000 (0.005%)	CM1413447 (SCD)	VUS	LP	VUS
104	ACM	PKP2	c.1130T>C	p.(Ile377Thr)	rs397516985	1/31416 (0.003%)	NA	VUS	LP	VUS

Table 1 (Continued)

Index case	Disease	Gene	Nucleotide	Protein	dbSNP	gnomAD	HGMD (disease)	ClinVar	2010 classification	2020 classification (ACMG)
105	SCD	SCN5A	c.287T>C	p.(Leu96Pro)	NA	NA	NA	NA	LP	VUS
106	ACM	PKP2	c.76G>A	p.(Asp26Asn)	rs143004808	1427/168710 (0.8%)	CM061172 (ACM)	LB	LP	LB
107	ACM	DSG2	c.908C>T	p.(Ser303Phe)	rs757792714	2/249400(0.0008%)	CM161318 (ACM)	VUS	LP	VUS
		DSC2	c.907G>A	p.(Val303Met)	rs145560678	282/282824 (0.09%)	CM117222 (DCM)	LB	LP	VUS
108	SCD	KCNE3	c.248G>A	p.(Arg83His)	rs17215437	859/282668 (0.3%)	CM011795 (PP)	LB	VUS	VUS
109	CPVT	RYR2	c.12419G>A	p.(Gly4140Glu)	NA	NA	NA	NA	LP	VUS
110	ACM	PKP2	c.253_256delCAGT	p.(Glu85Metfs**26)	rs786204388	1/251410(0.0003%)	CD102829 (ACM)	P	P	P
111	BrS	SCN5A	c.2924G>C	p.(Arg975Pro)	NA	NA	NA	NA	LP	VUS
112	SCD	RYR2	c.7201C>T	p.(Arg2401Cys)	rs1321283106	2/31398 (0.006%)	NA	NA	LP	VUS
113	HCM	MYBPC3	c.1513_1515delAAG	p.(Lys505del)	rs727504287	2/249264(0.0008%)	CD031519 (HCM)	NA	P	LP
		MYBPC3	c.565G>A	p.(Val189Ile)	rs11570052	637/245140 (0.25%)	CM169151 (HCM)	LB	LP	LB
114	BrS	SCN5A	c.1097T>C	p.(Phe366Ser)	NA	NA	NA	NA	LP	VUS
115	HCM	MYH7	c.5117T>C	p.(Leu1706Pro)	rs797044602	NA	CM042428 (Myopathy)	LP	VUS	VUS
116	HCM	MYH7	c.4865T>C	p.(Leu1622Pro)	NA	NA	NA	NA	VUS	VUS
117	SCD	KCNE2	c.22A>G	p.(Thr8Ala)	rs2234916	1059/282720 (0.37%)	CM003449 (LQTS, DI)	LB	LB	LB
118	ACM	DSP	c.88G>A	p.(Val30Met)	rs121912998	358/239538 (0.14%)	CM063961 (ACM)	LB	LP	LB
119	ACM	PKP2	c.175C>T	p.(Gln59*)	NA	NA	CM1313041 (ACM)	NA	LP	P
120	DCM	LMNA	c.1056G>T	p.(Met352Ile)	NA	NA	NA	NA	VUS	VUS
121	LQTS	KCNH2	c.982C>G	p.(Arg328Gly)	rs199473505	161/282246 (0.057%)	NA	VUS	VUS	VUS

Note – ACM: arrhythmogenic cardiomyopathy, AF: atrial fibrillation, ACMG: American College of Medical Genetics and Genomics, B: benign, BrS: Brugada syndrome, ClinVar: clinical variation, CPVT: catecholaminergic polymorphic ventricular tachycardia, DCM: dilated cardiomyopathy, DM: disease mutation, gnomAD: genome aggregation database, HCM: hypertrophic cardiomyopathy, HGMD: human genome mutation database, LB: likely benign, LP: likely pathogenic, LQTS: long QT syndrome, LQTS-DI: long QT syndrome drug-induced, MI: myocardial infarction, NA: not available data, P: pathogenic, PCCD: progressive cardiac conduction disease, PP: periodic paralysis, SCD: sudden cardiac death, SIDS: sudden infant death syndrome, SSS: sick sinus syndrome, SUNDS: sudden unexpected death syndrome, VUS: variant of uncertain significance.

genetic cause of IAS allows for accurate clinical diagnosis, risk stratification, adoption of personalized therapeutic measures, and early identification of relatives at risk, while also determining no genetic carriers [16]. Obtaining reliable and accountable interpretations of variant significance is as important as improving molecular diagnostic techniques, and for these reason adequate guidelines, and also a periodic update of the criteria used for interpretation and revision of the variant significances, are fundamental.

In our retrospective study, we have applied current ACMG recommendations for variant classification in a cohort of patients diagnosed with IAS and post-mortem cases with suspected IAS [4]. We determined that over 70% of variants required reclassification after ten years under the updated guidelines. Specifically, after reanalysis of the variants, 69.23% of diagnosed IAS cases required a change in variant classification. In the post-mortem cohort, 94.11% required a change in variant classification. This data reinforces the need for clinical data in genetic diagnoses; a complete clinical history contributes to variant classification and helps to clarify the role of a variant in each patient. Recently, a study focused on the reclassification of VUS in IAS concluded that disease-specific phenotypes significantly increase the accuracy of classification [10]. Interestingly, we determined that many missense variants changed their classification from LP to VUS after reanalysis; this modification is mainly due to the increase of items used in ACMG recommendations. As mentioned above, increase in items implies more accuracy in classification but also stringency. In contrast, many radical variants changed from LP to P, accordingly to Harrison *et al.* [17] This fact suggests that variants resulting in a premature truncation of proteins and/or frameshifts should be considered highly damaging and therefore should be carefully analysed. Missense variants should be comprehensively analysed in each patient, considering all available data to perform a proper variant prioritization in a personalized clinical context [18]. Family segregation is the most robust tool to corroborate the pathogenic role of a particular variant. Unfortunately, a complete segregation for most rare variants currently associated with IAS is not available. In addition, incomplete penetrance/variable expressivity are hallmarks of IAS, so a segregation analysis of at least three generations should be recommended to obtain helpful data [19, 20]. Thus, the disease manifestation observed at the basal assessment and the clinical evolution on the follow-up, not only in the index cases but in the entire families, may also be highly useful in the understanding of the pathogenic role of the initially identified variants. Despite all the previously mentioned considerations, the frequency of rare variants in the global population is the first tool used to help to discern a potential damaging variant from other rare variants with no potential deleterious role. Nowadays, free and quick access to on-line databases focused on variant population frequencies makes this an easy routine approach.

Classification of a variant as VUS or downgrading a variant status from LP to VUS does not mean that there is less pathogenic risk of IAS for any patient who carries the rare variant; ambiguous significance implies that current evidence does not back a conclusive deleterious role in IAS. Therefore, clinical translation of VUS should be performed with caution and VUS should not be discarded, at least until additional data becomes available focused on clarifying their clinical role. Current recommendations for the interpretation of rare variants [4] include more items to be considered than ten years ago [11]. This increase in items implies more accuracy in classification but also increased stringency; thus, a lack of data for some of these items leads to ambiguous classification [5]. As a consequence, a low percentage of variants classified currently as VUS confer a real of risk in IAS and most are LB [21]. To discriminate a true risk-carrying variant from a non-deleterious variant is a challenge without accurate family segregation and functional studies [7]. Expected frequencies of each IAS variant and constant update of minor allele frequencies in large global population studies should be used to help identify the genes,

Table 2
Modifications in classification of rare variant.

	Total	Total of Changes	PM	Changes in PM	CL	Changes in CL
Rare Variants	128	92 (71,87%)	17	16 (94,11%)	111	76 (68,46%)
Cases	121	88 (72,72%)	17	16 (94,11%)	104	72 (69,28%)

Note − CL: clinical group, PM: post-mortem group.

regions of genes, and/or types of variants strongly associated with IAS which may help to determine the roles of variants in clinical settings, particularly if they are classified as VUS [22].

At the present time, while the ACMG recommend how to classify variants, there is currently no consensus for when and how often variants should be reclassified. Therefore, reinterpretation of genetic variants occurs mainly due to a clinician's request, identification of a previously classified variant in a new patient or new data available concerning the rare variant [23]. These expectations should be explicitly delineated as part of the informed consent process before the sample is obtained and reviewed again when disclosing initial results [24]. Concerning IAS, Smith *et al.* reported a reclassification after one year of 3% of rare variants [25]. In 2018, a reclassification of rare variants previously considered deleterious in Brugada Syndrome was performed; only 37% were classified as P or LP following current ACMG recommendations [8]. A recent study identified a modification in 52% of rare variants classified as VUS seven years ago [10]. Therefore, the evidence supports the periodic reclassification of the rare variants in IAS despite lack of data concerning the time of re-evaluation. In our report, more than 70% of rare variants were reclassified after ten years, also supporting the necessity of re-evaluation. In the light of all this evidence, we propose that rare variants associated with IAS should be reanalysed within five years if already classified following ACMG recommendations since it seems to be adequate to manage the rapid obsolescence of genetic data interpretations. In addition, our results support further urgent reanalysis of each IAS rare variant if they were not classified originally following ACMG recommendations.

The next step should be clinical translation of the re-evaluation and assessment of the implications in families because the confirmation of a variant as P, or removal of a P designation, may have a significant impact on patients and relatives. Therapeutic management can
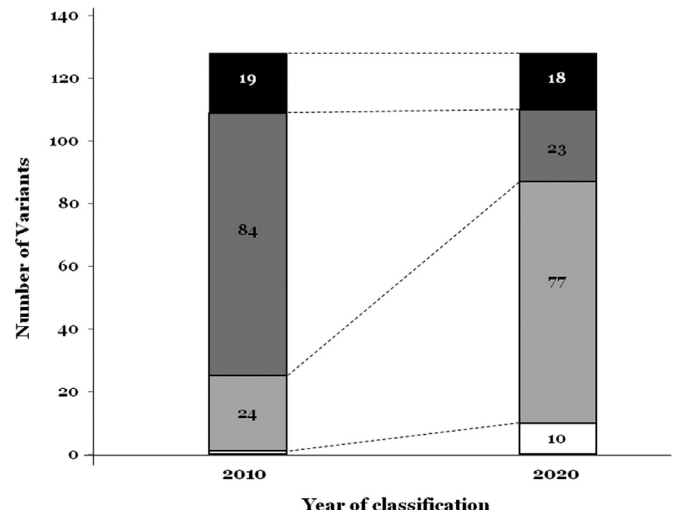

Fig. 2. Reclassification of rare variants. White colour represents the number of rare variants classified as LB. Soft grey colour represents the number of rare variants classified as VUS. Dark grey colour represents the number of rare variants classified as LP. Black colour represents the number of rare variants classified as P. Concrete number of variants is included inside each part.

be modified but emotional and psychological impacts may have lasting effects [26]. New genetic information can lend itself to misinterpretation [5], so we recommended discussions with an expert cardiologist in genetics to explain what reclassification entails for each patient, accordingly to recent American Society of Human Genetics recommendations (ASHG) [27]. One key point is that a change in classification does not necessarily change the fact that a case has an IAS. Finally, it is important to remark that our re-information approach to families follows the ethical premise that definitive consideration for any clinical or research guideline should be improving patient medical care.

We can highlight some major limitations to our study. Variant interpretation is subject to inherent intra- and inter-laboratory differences in data interpretation [28]. In the current study, three of the authors performed independent classification following ACMG recommendations and all authors came to a consensus regarding the final classification decision. All rare variants were identified after a

Table 3
Classification of rare variants in each gene.

Genes	Number variants	2010				2020				Modification 2010 *vs.* 2020
		LB	VUS	LP	P	LB	VUS	LP	P	
DES	1	.	1	.	.	.	1	.	.	NO
DSC2	2	.	.	2	.	.	2	.	.	YES
DSG2	8	.	3	5	.	2	5	1	.	YES
DSP	4	.	1	3	.	2	1	.	1	YES
JUP	3	.	3	.	.	.	3	.	.	NO
KCNE1	1	.	.	1	.	1	.	.	.	YES
KCNE2	2	.	2	.	.	2	.	.	.	YES
KCNE3	1	.	1	.	.	.	1	.	.	NO
KCNH2	9	.	2	5	2	.	7	2	.	YES
KCNQ1	6	.	1	1	4	.	1	5	.	YES
LMNA	1	.	1	.	.	.	1	.	.	NO
MYBPC3	4	.	.	3	1	1	.	1	2	YES
MYH7	4	.	4	.	.	.	4	.	.	NO
PKP2	17	.	2	8	7	2	3	4	8	YES
RYR2	9	.	.	9	.	.	9	.	.	YES
SCN5A	55	1	3	46	5	.	38	10	7	YES
TGFB3	1	.	.	1	.	.	1	.	.	YES
TOTAL	17	128	1	24	84	19	10	77	23	18	

Note − LB: likely benign, LP: likely pathogenic, P: pathogenic, VUS: variant of uncertain significance.

Table 4
Classification of rare variants in IAS subtypes.

Disease	Number Variants		All 2010	All 2020	Missense 2010	Missense 2020	Radical 2010	Radical 2020
BrS	49	LB	1	.	.	.	1	.
		VUS	3	34	3	33	.	1
		LP	41	8	30	2	11	6
		P	4	7	2	0	2	7
LQTS	12	LB	.	.	.	.	.	.
		VUS	3	4	3	4	.	.
		LP	3	8	2	7	1	1
		P	6	.	6	.	.	.
CPVT	4	LB	.	.	.	.	.	.
		VUS	.	4	.	4	.	.
		LP	4	.	4	.	.	.
		P	.	.	.	.	.	.
ACM	36	LB	.	6	.	6	.	.
		VUS	10	16	10	16	.	.
		LP	19	5	12	1	7	4
		P	7	9	1	.	6	9
DCM	1	LB	.	.	.	.	.	.
		VUS	1	1	1	1	.	.
		LP	.	.	.	.	.	.
		P	.	.	.	.	.	.
HCM	8	LB	.	1	.	1	.	.
		VUS	4	4	4	4	.	.
		LP	3	1	1	.	2	1
		P	1	2	.	.	1	2

Note – ACM: arrhythmogenic cardiomyopathy, BrS: Brugada syndrome, CPVT: cate-cholaminergic polymorphic ventricular tachycardia, DCM: dilated cardiomyopathy, HCM: hypertrophic cardiomyopathy, LB: likely benign, LQTS: long QT syndrome, LP: likely pathogenic, P: pathogenic, VUS: variant of uncertain significance.

limited analysis of genes; we cannot be sure that patients do not carry other rare variants in genes currently unassociated with IAS. Only genes currently associated with IAS were analysed. The number of cases analysed was small, so comprehensive reassessment should be performed in large cohorts of IAS samples to corroborate a periodic reclassification. Finally, lack of data for some of the rare variants, mainly concerning functional studies as well as familial segregation, impedes comprehensive interpretation of our results and definite classification.

In summary, reanalysis using current ACMG recommendations showed that 71.87% of rare variants in IAS were given a new classification than originally assigned ten years ago. Many variants, however, remain of ambiguous significance. These findings emphasize the importance of cautious interpretation of variant scoring and comprehensive family segregation, supporting the periodic re-evaluation of rare variants in IAS before clinical translation. It is extremely important that in cases of significance changes after an update the geneticist promptly informs the interested patients.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Author's contributions

Conceptualization_OC, GS, AO, JB, and RB, Data curation_OC, AF, MC, AI, EA, SG, AG, PJ, and RT, Formal analysis_OC, AF, MC, AI, CF, SC, EA, and SG, Funding acquisition_OC, GS, JB, and RB, Investigation_OC, AF, MC, PJ, and RT, Methodology_OC, SC, EA, SG, AG, PJ, and RT, Project administration_OC, GS, AO, JB, and RB, Resources_OC, GS, AO, JB, and RB, Software_CF, SC, Supervision_OC, GS, AO, JB, and RB, Validation_OC, GS, AO, JB, and RB, Visualization_OC, GS EA, JB, and RB, Writing-original draft_OC, GS, AO, JB, and RB, Writing-review & editing_OC, GS, AO, JB, and RB.

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