

# Functionally validated SCN5A variants allow interpretation of pathogenicity and prediction of lethal events in Brugada syndrome

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## Aims

The prognostic value of genetic variants for predicting lethal arrhythmic events (LAEs) in Brugada syndrome (BrS) remains controversial. We investigated whether the functional curation of SCN5A variations improves prognostic predictability.

## Methods and results

Using a heterologous expression system and whole-cell patch clamping, we functionally characterized 22 variants of unknown significance (VUSs) among 55 SCN5A mutations previously curated using *in silico* prediction algorithms in the Japanese BrS registry ( $n = 415$ ). According to the loss-of-function (LOF) properties, SCN5A mutation carriers ( $n = 60$ ) were divided into two groups: LOF-SCN5A mutations and non-LOF SCN5A variations. Functionally proven LOF-SCN5A mutation carriers ( $n = 45$ ) showed significantly severer electrocardiographic conduction abnormalities and worse prognosis associated with earlier manifestations of LAEs (7.9%/year) than *in silico* algorithm-predicted SCN5A carriers (5.1%/year) or all BrS probands (2.5%/year). Notably, non-LOF SCN5A variation carriers ( $n = 15$ ) exhibited no LAEs during the follow-up period. Multivariate analysis demonstrated that only LOF-SCN5A mutations and a history of aborted cardiac arrest were significant predictors of LAEs. Gene-based association studies using whole-exome sequencing data on

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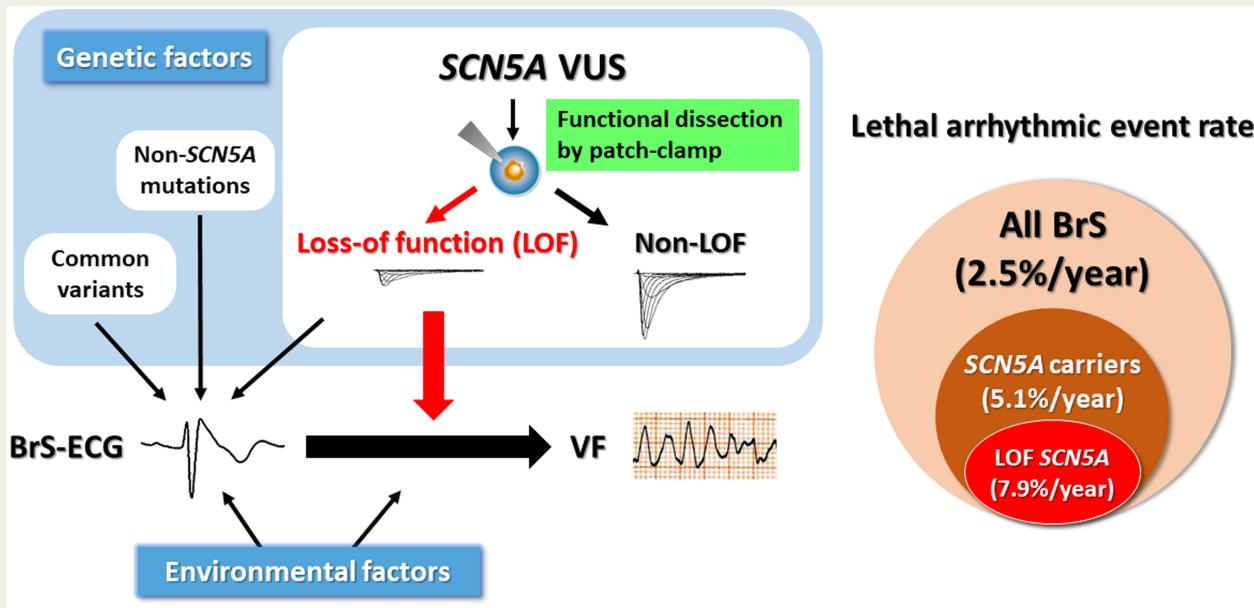
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another independent SCN5A mutation-negative BrS cohort ( $n=288$ ) showed no significant enrichment of rare variants in 16 985 genes including 22 non-SCN5A BrS-associated genes as compared with controls ( $n=372$ ). Furthermore, rare variations of non-SCN5A BrS-associated genes did not affect LAE-free survival curves.

## Conclusion

*In vitro* functional validation is key to classifying the pathogenicity of SCN5A VUSs and for risk stratification of genetic predictors of LAEs. Functionally proven LOF-SCN5A mutations are genetic burdens of sudden death in BrS, but evidence for other BrS-associated genes is elusive.

## Graphical Abstract



Among currently recognized genetic and non-genetic risk factors for Brugada syndrome, functionally validated loss-of-function SCN5A mutations are associated with worse prognosis with earlier manifestation of lethal arrhythmic events (7.9%/year) than *in silico* algorithm-predicted SCN5A rare variations (5.1%/year) or Brugada-type ECG (2.5%/year).

## Keywords

Brugada syndrome • SCN5A mutations • Lethal arrhythmia • Variants of unknown significance • Whole-exome sequencing • Patch-clamp

## Translational Perspective

SCN5A mutations are associated with the risk of lethal arrhythmia in Brugada syndrome (BrS); however, nearly 70% of BrS-associated SCN5A rare variations registered in ClinVar are classified as variants of unknown significance, requiring curation strategies to accurately differentiate pathogenic from benign variations to predict patient prognosis. As a monogenic trait, functionally validated loss-of-function SCN5A mutations, but not rare coding variants of other BrS-associated genes, are genetic risks of lethal arrhythmias in BrS. Although the contribution of polygenic factors in BrS warrants further investigation, these results may help to develop a new personalized risk stratification paradigm for sudden cardiac death.

## Introduction

Brugada syndrome (BrS) is a rare heritable arrhythmia characterized by coved-type ST-segment elevation in the right precordial leads and an increased risk of sudden cardiac death due to lethal ventricular

arrhythmia.<sup>1,2</sup> Mutations in the SCN5A gene, encoding cardiac sodium channel (Nav1.5), are identified in approximately 20% of cases; however, the predictive value of SCN5A mutations for subsequent lethal arrhythmic events (LAEs) remains controversial. Specifically, SCN5A mutations were not associated with LAE in European BrS cohorts,<sup>3,4</sup>

whereas our Japanese multicentre BrS cohort study has previously demonstrated that 60 probands carrying 55 different *SCN5A* mutations exhibited their first LAE at younger ages (34 vs. 42 years) than probands without *SCN5A* mutations ( $n=355$ ).<sup>5</sup> Observations consistent with those of the latter study were reported in an Italian BrS cohort.<sup>6</sup> The selection bias of patients<sup>5</sup> or demonstrated transethnic differences in the phenotypic severity of BrS, and the frequency of *SCN5A* variations<sup>7</sup> may underlie the discrepancy of the aforementioned studies. Nevertheless, and more importantly, *SCN5A* mutations often exhibit incomplete penetrance in BrS, and this gene is associated with a relatively high background rate of rare missense variants in the general population (2–5%). Moreover, efforts to enhance the classification of *SCN5A* missense variants using protein topology-driven estimated predictive assessments or *in silico* prediction algorithms are limited compared with those for other major cardiac channelopathy genes, such as *KCNQ1*/*KCNH2*.<sup>8</sup> Therefore, risk assessment of *SCN5A* variants using phenotypic data of a single variant carrier remains challenging, and an inaccurate classification of rare variants might have obscured the prognostic value of *SCN5A*.

The American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) guidelines provide approaches for a more appropriate classification of pathogenic variants, and ‘functional studies supporting a deleterious effect (PS3)’ are assigned one of four criteria with ‘strong evidence’ of pathogenicity.<sup>9</sup> Loss of function (LOF) of cardiac Na current ( $I_{Na}$ ) due to *SCN5A* mutations is the primary pathophysiology underpinning BrS. A previous functional study has shown that patients carrying null *SCN5A* mutations experience more frequent episodes of syncope and more severe conduction abnormalities than those with other types of *SCN5A* mutations.<sup>10</sup> More recently, Glazer *et al.* have shown that patch clamping enables reclassification of variants of unknown significance (VUSs) of *SCN5A*.<sup>11</sup> These studies suggest that *in vitro* functional re-evaluation of *SCN5A* variations may improve LAE predictability in BrS. In the present study, we conducted a PubMed search and re-evaluated the function of 55 *SCN5A* rare variants previously curated via multiple *in silico* prediction algorithms in a Japanese BrS cohort<sup>5</sup> and determined whether the functionally proven *SCN5A* mutations may improve the predictive value for LAE in patients with BrS.

Increasing evidence suggests that BrS is unlikely to be a Mendelian monogenic disease but rather an oligogenic disorder involving multiple rare and nonrare variants, as well as structural abnormalities and inflammation, contributing to the underlying basis of the disease.<sup>12</sup> A previous international genome-wide association study identified three common independent susceptibility variants close to *SCN5A*, *SCN10A*, and *HEY2*,<sup>13</sup> and to date, >20 genes have been reported to be associated with BrS.<sup>14</sup> Although no significant enrichment of these rare coding variants, except for *SCN5A*, were observed in BrS cases<sup>15</sup> and the ClinGen consortium recently reported *SCN5A* as the only causative gene with definitive evidence for the diagnosis of BrS,<sup>14</sup> the predictive value of non-*SCN5A* coding variants for the long-term prognosis of BrS—i.e. LAE and sudden cardiac death—has never been evaluated. In this study, we performed whole-exome sequencing on a distinct *SCN5A*-negative BrS cohort to identify novel

pathological rare variants and assessed if non-*SCN5A* rare coding variants contribute to the genetic burden of sudden death in BrS.

## Methods

### Patients and study cohorts

The diagnosis of BrS was made according to the criteria of a consensus report,<sup>16</sup> and LAE was defined as sudden cardiac death, cardiac arrest, ventricular tachycardia/ventricular fibrillation, or appropriate discharge of implantable cardioverter defibrillator (ICD). Clinical characteristics including time to first LAE and electrocardiographic (ECG) parameters were obtained as previously described.<sup>5</sup> This study was approved by the institutional review board (National Cerebral and Cardiovascular Center, R19048) and local ethics committee of each institution. All participants in the cohorts provided written informed consent before clinical and genetic investigations in accordance with the Declaration of Helsinki.

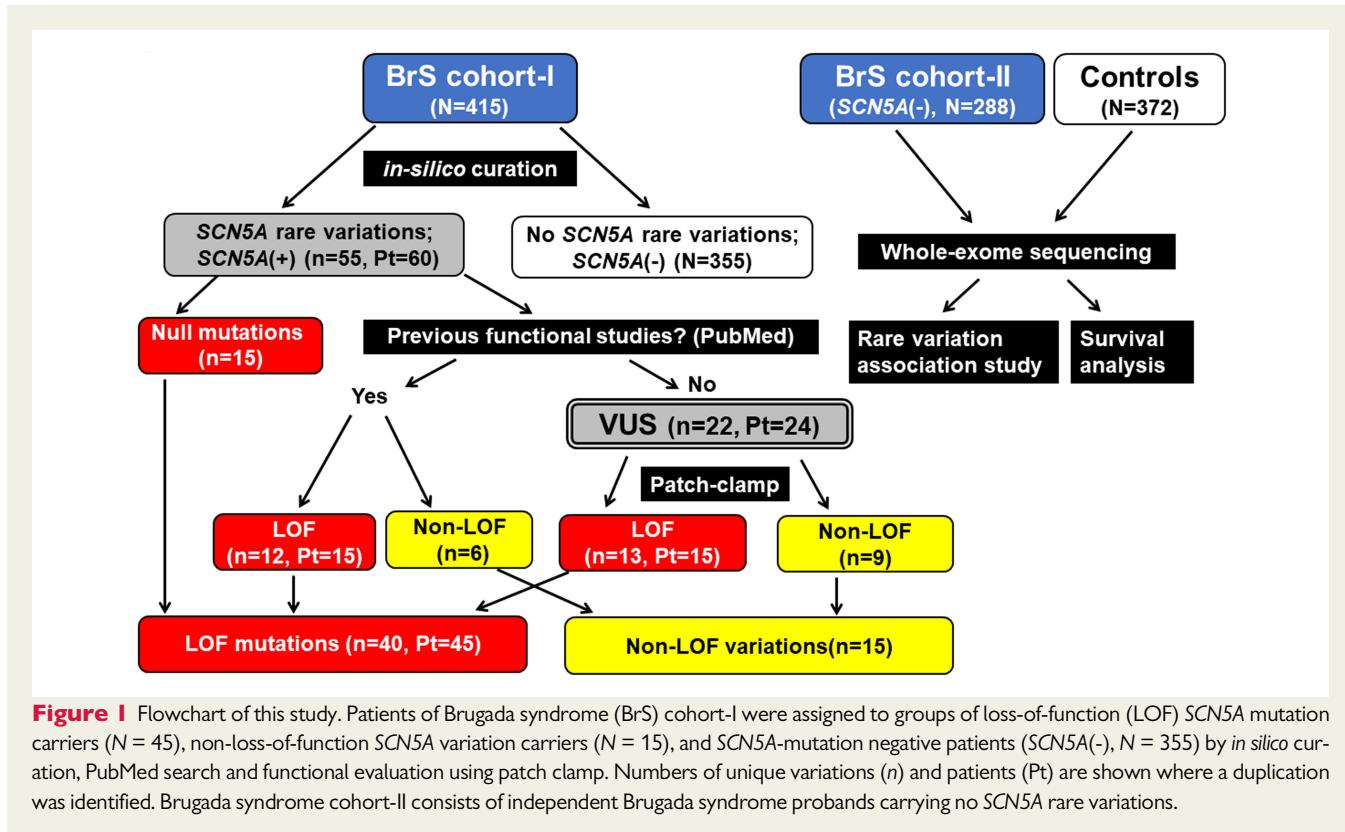
This study consisted of two independent Japanese multicentre BrS cohorts, specifically BrS cohort-I (415 probands)<sup>5</sup> and BrS cohort-II (288 unrelated probands), and 372 ethnic-matched controls (Figure 1 and Table 1). In BrS cohort-I, BrS probands were assigned as *SCN5A* mutation carriers (*SCN5A*(+);  $n=60$ ) and *SCN5A* mutation negative probands (*SCN5A*(-);  $n=355$ ) based on Sanger sequencing as previously described.<sup>5</sup> We enrolled independent Japanese BrS probands (cohort-II), whose negative *SCN5A* genotype statuses were determined in advance by Sanger sequencing. BrS cohort-II and the control Japanese subjects were subjected to whole-exome sequencing and gene-wise association test. *In silico* prediction of *SCN5A* variants was performed using seven algorithms as previously described.<sup>5,8</sup> Further information is provided in the Supplementary material online, Methods.

### Assignment and functional evaluation of 22 variants of unknown significance

We performed public database screening and PubMed literature search for 55 variations reported in BrS cohort-I and identified 22 VUSs. To functionally evaluate the Na channel properties of 22 VUSs, we constructed human *SCN5A* expression plasmids of VUSs using site-directed mutagenesis, and  $I_{Na}$  of HEK293T cells were recorded using the whole-cell patch-clamp technique using a heterologous expression system. After analysing the biophysical properties of 22 VUS channels, the 55 variants were categorized into two groups, LOF and non-LOF, according to the presence or absence of significantly reduced peak  $I_{Na}$  density than wild-type (WT) *SCN5A*, respectively. Detailed information is provided in the Supplementary material online, Methods.

### Statistical analyses

Quantitative variables are shown as mean  $\pm$  standard deviation unless otherwise stated. Statistical significance was set at  $P < 0.05$ . For the statistical analysis of continuous variables with a normal distribution, one-way analysis of variance followed by Bonferroni’s post hoc comparison tests was used. The cumulative probability of an index LAE over the course of the patient follow-up or their entire lifetime was determined using Kaplan–Meier methods for each subgroup, and the difference in survival rates was analysed using a log-rank test. Univariate analysis using a Cox proportional-hazards model was performed to determine variables that improve the prediction of LAEs. Independent variables with  $P < 0.05$  in the univariate analyses were included in the multivariate analysis. Statistical analyses were performed using the R programme (version 4.0.2) and SPSS statistical package (version 26).



## Results

### Functional classification of BrS-associated 55 SCN5A variations

A PubMed search identified 21 publications that described the biophysical properties of 22  $SCN5A$  variations (17 missense, one in-frame deletion, and four nonsense variations) (Figure 1 and *Supplementary material online*, *Table S1*). Moreover, among 55 variations, 11 were novel null variants (two nonsense, six frame-shift, three canonical splice site) classified as PVS1 (very strong evidence of pathogenicity) according to the ACMG-AMP guidelines.<sup>9</sup> Accordingly, the remaining 22 missense variations were assigned as VUS (Table 1). The functional properties of each VUS were analysed using whole-cell patch-clamp assays (*Supplementary material online*, *Figure S1*) and categorized into two groups according to the degree of peak  $I_{Na}$  reduction: LOF (significantly reduced  $I_{Na}$  density compared with WT;  $n = 13$ ), and non-LOF (no significant difference compared with WT;  $n = 9$ ), and the border zone of LOF and non-LOF was 53.2–65.6% (*Supplementary material online*, *Table S2*). Since the experimental conditions of current study and previous patch-clamp studies were largely similar (*Supplementary material online*, *Table S3*), both data were combined with null PVS1 mutations ( $n = 11$ ) to classify a total of 55 variants according to their biophysical properties as follows: LOF ( $n = 40$ ) and non-LOF ( $n = 15$ ) (Figure 2 and *Supplementary material online*, *Table S4*). The locations of the 55 variants, illustrated based on Nav1.5 protein topology, exhibited diffuse distribution within the entire protein.

**Table I** Clinical characteristics of two independent Japanese Brugada syndrome cohorts

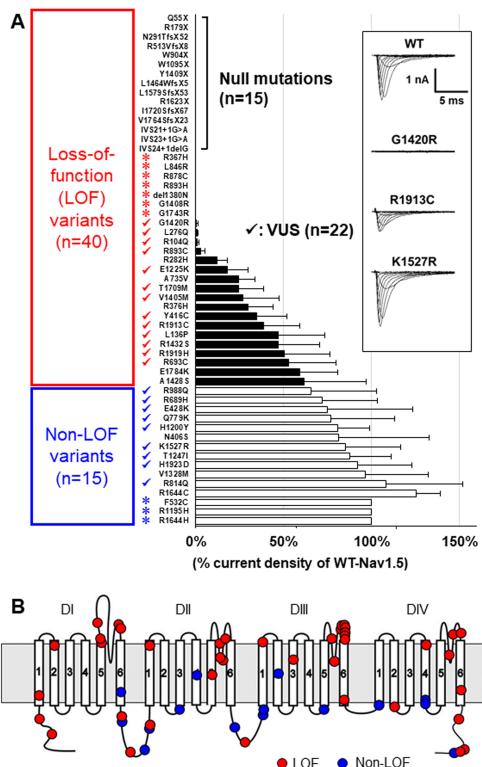
	BrS cohort-I	BrS cohort-II
Total ( $n$ )	415	288
Male sex ( $n$ , %)	403 (97.1)	274 (95.1)
Age at diagnosis [mean $\pm$ SD (median)]	46.1 $\pm$ 13.8 (44.9)	42.6 $\pm$ 14.2 (41.0) <sup>a</sup>
Baseline type-1 ECG ( $n$ , %)	299 (72.0)	210 (72.9)
$SCN5A$ rare variant carriers ( $n$ , %)	60 (14.5)	0 (0)
History of LAE ( $n$ , %)	88 (21.2)	180 (62.5)
History of non-LAE symptoms ( $n$ , %)	99 (23.9)	102 (35.4)
Asymptomatic ( $n$ , %)	228 (54.9)	6 (2.1)

BrS, Brugada syndrome; ECG, electrocardiography; LAE, lethal arrhythmic event; SD, standard deviation.

<sup>a</sup>Patients lacking age information ( $n = 49$ ) were excluded.

### Correlation between functional severity of SCN5A variations and electrocardiographic parameters

Among different ECG parameters, cardiac conduction properties (P, QRS, S durations, and PQ interval) were significantly prolonged in



**Figure 2** Functional classification of 55 *SCN5A* rare variations of Japanese Brugada syndrome cohort-I. (A) Whole-cell currents of rare *SCN5A* variants and wild-type (WT) Nav1.5 channel were recorded (inset and [Supplementary material online, Figure S1](#)) from HEK293T cells, and the percentage peak current densities vs. wild type were plotted. Variants were classified into loss of function (significantly reduced peak current density than wild type;  $n = 40$ ) or non-loss of function (not significantly different from wild type;  $n = 15$ ). Check marks indicate variants of unknown significance (VUS) for which patch-clamp was performed in this study ( $n = 22$ ), and asterisks represent variations of previous literatures whose precise current density data are unavailable ( $n = 10$ ). (B) Location of 55 *SCN5A* variants of loss of function and non-loss of function are shown with topological representation of Nav1.5.

the LOF compared to the non-LOF or *SCN5A*(-) ([Supplementary material online](#), *Figure S2*). However, no significant differences were observed in these parameters between non-LOF and *SCN5A*(-), suggesting that conduction parameters reflect the severity of Na channel dysfunction associated with *SCN5A* variations ([Supplementary material online](#), *Table S5*). Alternatively, other electrophysiological and clinical findings were largely comparable among the three groups ([Supplementary material online](#), *Figure S3*).

## Lethal arrhythmic events associated with the severity of sodium channel dysfunction

A total of 62 probands (15%) developed LAEs during the mean follow-up period of 72 months. Notably, none of the non-LOF

probands developed LAEs during follow-up (*Figure 3*). Furthermore, LOF exhibited significantly more frequent total lifetime events and ICD implantation than non-LOF. Most LAEs ( $n = 56$ , 90%) were terminated by appropriate ICD discharges, and LAE-free rates by Kaplan–Meier analysis were comparable regardless of ICD discharge. These data suggest that an appropriate ICD discharge serves as a surrogate for sudden cardiac death in BrS ([Supplementary material online](#), *Figure S4*); therefore, it was hypothesized that the prognosis of BrS patients can be discriminated based on LOF properties of the SCN5A variants.

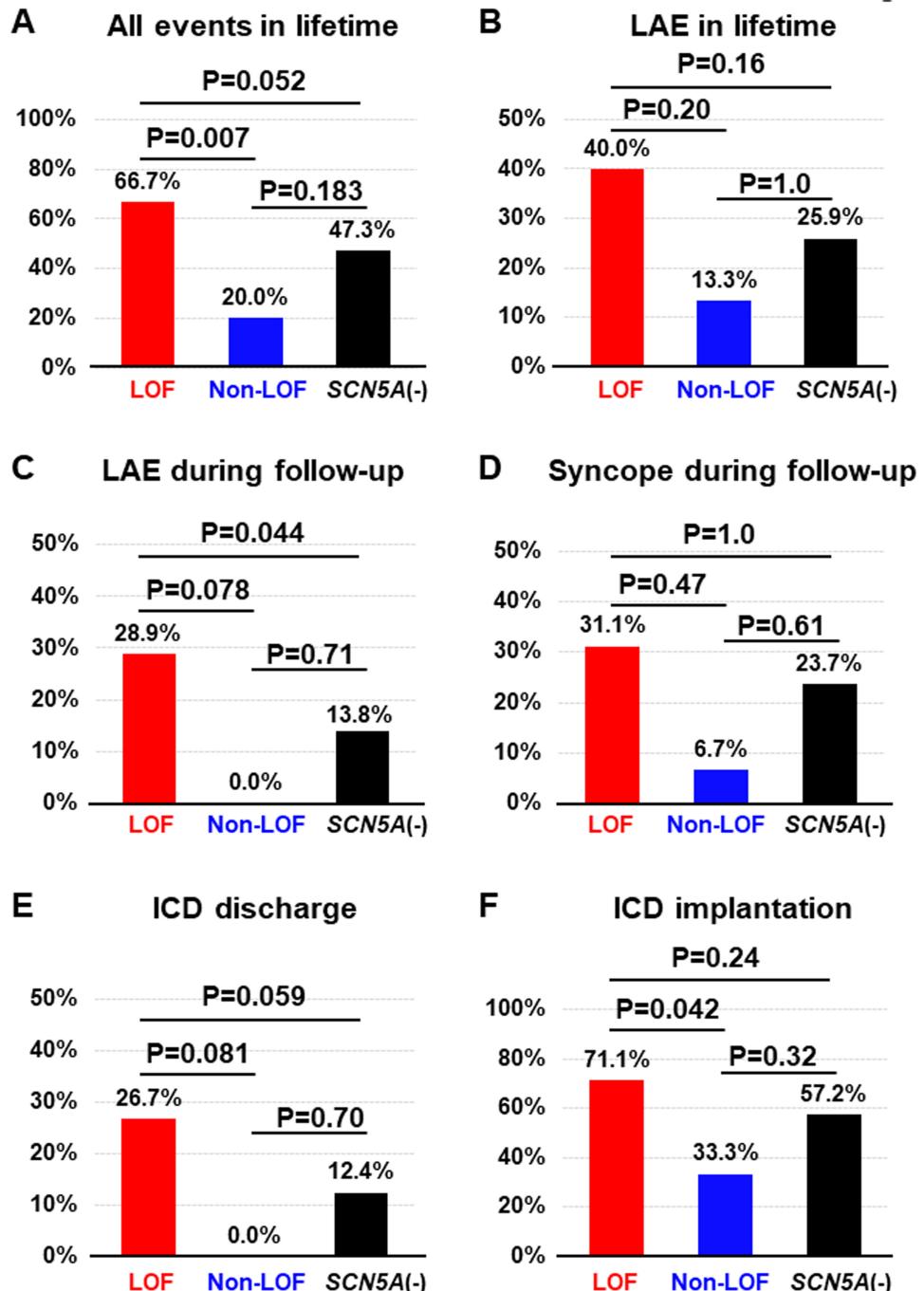
Based on this assumption, we calculated the cumulative rate of an index LAE during the mean follow-up period of 72 months (range, 1–249 months) using the Kaplan–Meier method for several subgroups with different statuses with respect to Na channel properties (Figure 4). Patients carrying *SCN5A* rare variants (*SCN5A*(+);  $n = 60$ ) had a significantly higher annual LAE rate than *SCN5A*(-) ( $n = 355$ ; 5.1%/year vs. 2.2%/year;  $P = 0.017$ , Figure 4A and Table 2), as previously reported.<sup>5</sup> The estimated mean LAE-free periods (mean  $\pm$  standard error) for patients of *SCN5A*(+) and *SCN5A*(-) were  $136.6 \pm 12.9$  and  $210.8 \pm 6.0$  months, respectively. As shown in Figure 4B, none of the non-LOF subgroup developed LAEs during the follow-up period, whereas the LOF subgroup had a significantly higher LAE rate (7.9%/year,  $P = 0.019$ ; Figure 4B, Table 2, and Supplementary material online, Table S6) and a shorter LAE-free period ( $94.5 \pm 10.7$  months). By combining non-LOF and *SCN5A*(-) results, we re-evaluated the survival curves of patients with or without LOF-*SCN5A* mutations (Figure 4C) and found that the LOF subgroup exhibited a significantly higher annual LAE rate and shorter estimated mean LAE-free period than the non-LOF plus *SCN5A*(-) (2.1%/year,  $208.8 \pm 5.9$  months,  $n = 370$ ;  $P = 0.0001$ , Figure 4C and Table 2). Qualitatively similar results were obtained from Kaplan–Meier analysis with shorter follow-up period (<107 months) (Supplementary material online, Figure S5).

## Reclassification of *SCN5A* variations and the predictability of LAEs

Univariate analysis using a Cox proportional hazard model showed that the positive status of both functionally validated LOF-SCN5A mutations and *in silico* algorithm-predicted rare SCN5A variations are significant predictors of LAEs, but the former exhibited a higher hazard ratio than the latter (Table 3 and [Supplementary material online](#), Table S6).<sup>5</sup> Multivariate analyses were then performed using independent variables with  $P < 0.05$  in univariate analysis (with two different SCN5A statuses) (Tables 3 and 4). A history of aborted cardiac arrest was the strongest predictor of LAEs regardless of the SCN5A status. Moreover, SCN5A variant status was a significant predictor of LAE, and the predictive values of functionally validated LOF-SCN5A mutations were higher than that of *in silico*-predicted rare SCN5A variations demonstrated previously.<sup>5</sup> In contrast, prolonged QRS, or documented atrial fibrillation, were not predictors of LAEs in BrS.

## Genome-wide screening and risk stratification of BrS-associated genes other than *SCN5A*

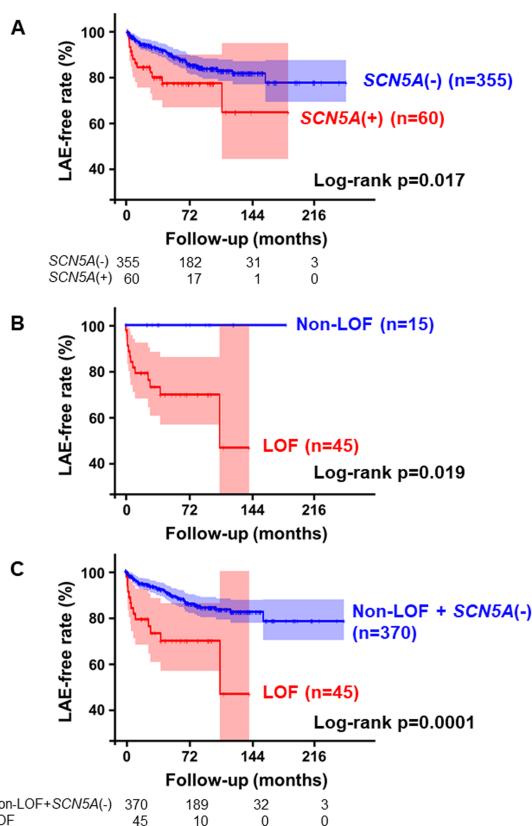
To determine which genes besides *SCN5A* carry burden of rare genetic variations in BrS cases vs. controls, we performed whole-exome sequencing on a distinct Japanese cohort of *SCN5A*(-) BrS (BrS



**Figure 3** Association of clinical events in Brugada syndrome patients with distinct Na channel function properties. (A) All events and (B) lethal arrhythmic events (LAE) in lifetime; (C) lethal arrhythmic events and (D) syncope during follow-up; (E) implantable cardioverter defibrillator (ICD) discharge and (F) implantable cardioverter defibrillator implantation were compared among Brugada syndrome patients with loss of function ( $n = 45$ ), non-loss of function ( $n = 15$ ), and SCN5A(-) ( $n = 355$ ). Statistical analysis was performed using Fisher's exact test with Bonferroni adjustment.

cohort-II,  $n = 288$ ) and controls ( $n = 372$ ). Then, we performed gene-wise association tests using rare variations using two different cut-off values of minor-allele frequency (<1% and <0.3%); however, we failed to identify novel genes significantly enriched with rare coding variations among the entire set of genes in BrS cohort-II

(Supplementary material online, Figure S6) or previously recognized 22 non-SCN5A BrS-associated genes<sup>14</sup> (Supplementary material online, Tables S7 and S8). We assessed whether rare coding variants of 22 BrS-associated genes with limited evidence modify the prognosis of BrS; lifetime cumulative LAE-free rates were calculated by



**Figure 4** Kaplan–Meier analysis of lethal arrhythmic event-free survival during follow-up in Brugada syndrome cohort-I. (A) Lethal arrhythmic event-free survival during the follow-up period in Brugada syndrome probands carrying SCN5A rare variations (all SCN5A;  $n = 60$ ) and SCN5A(-) ( $n = 355$ ). Confidence bands indicate 95% pointwise CI. (B) Time course of Brugada syndrome patients with loss-of-function SCN5A mutations ( $n = 45$ ) and non-loss of function ( $n = 15$ ). Non-loss-of-function probands have no lethal arrhythmic events during the follow-up period. (C) Lethal arrhythmic event-free survival of loss of function ( $n = 45$ ) vs. non-loss of function plus SCN5A(-) ( $n = 370$ ). The dissociation between two survival curves is more pronounced than that in panel A.

Kaplan–Meier method. However, log-rank tests showed that these rare variants did not affect the age of initial LAE in BrS cohort-II (Figure 5). Even when focusing on genes that are known to modulate cardiac Na channel function, rare variants of these genes were not enriched in cases nor affected the prognosis of BrS cohort-II (Supplementary material online, Figure S7 and Supplementary material online, Table S8). Thus, we find no evidence supporting an association between the BrS-associated non-SCN5A genes and sudden arrhythmic death.

## Discussion

In this study, we aimed to dissect the genetic basis of BrS by conducting electrophysiological evaluations of SCN5A variations and demonstrated that functionally validated LOF-SCN5A mutations, not rare

coding variations of other BrS-related genes, are associated with genetic risks of lethal arrhythmia in BrS. In addition to a history of aborted cardiac arrest being the strongest and most well-established predictor of future LAEs in patients with BrS, we demonstrated, to the best of our knowledge, for the first time that LOF-SCN5A mutations are an independent and significant predictor of sudden death.

Advances in genetic sequencing have increased the potential yield of genetic testing, while raising the clinical dilemma of the discovery of many VUSs compromising the accuracy of variant interpretation. The pathogenicity of SCN5A variants in BrS has often been unknown or disputed; 67.5% of the total 1140 BrS-associated SCN5A variations submitted to ClinVar are classified as either of uncertain significance, no assertion provided, or conflicting interpretations.<sup>17</sup> These VUSs are often specific to a particular family, and their penetrance and expressivity are highly variable in BrS,<sup>1</sup> hampering the annotation of their pathogenicity through segregation analysis. In the ACMG-AMP guidelines, the evidence level of pathogenicity for ‘absent in population databases’ is assigned as moderate (PM2), while that of ‘in silico prediction algorithms’ is assigned as supporting (PP3). Specificity of in silico algorithms to predict the pathogenicity of missense variants is generally low despite their high sensitivity,<sup>9</sup> resulting in the overprediction of missense variations as deleterious. Recent studies, using purely in silico analyses, including systematic evaluation using the ACMG-AMP guidelines, failed to predict the disease risk of SCN5A variants in BrS.<sup>18,19</sup> These results support the observation of our study that 27% of the SCN5A missense VUSs (15/55) were overpredicted in silico, therefore implicating the need for additional reliable tools to improve the annotation of pathogenicity for large numbers of SCN5A VUSs. In this study, we propose that the functional evaluation of SCN5A VUSs using a patch-clamp study might be an efficient strategy to aid the differentiation of malignant variants associated with predisposition to sudden death from those that are innocuous (Graphical Abstract).

Among the 55 functionally reclassified SCN5A variants, including 22 VUSs, most (40 variants, 73%, LOF) showed a significant reduction in peak  $I_{Na}$  compared to WT-SCN5A, which was associated with more severe abnormalities in ECG conduction parameters (Supplementary material online, Figure S2), and an earlier manifestation of LAEs (Figure 4). Note that our *in vitro* functional classification of SCN5A variants according to the significant  $I_{Na}$  density reduction (LOF vs. non-LOF) successfully dissected the cumulative risk of LAEs in the 60 carriers during the follow-up period (Figure 4B). The close relationship between the degree of SCN5A Na channel dysfunction and the phenotypic severity has been previously reported; SCN5A truncation mutation carriers were found to have more syncopal episodes and prolonged cardiac conduction abnormalities than missense mutation carriers.<sup>10</sup> Another Italian study of 92 BrS patients identified four SCN5A mutations (R104Q, L276Q, E122K, and A142S) in 12 patients with LAE during follow-up,<sup>6</sup> and our dataset included BrS probands carrying the identical LOF mutations (Supplementary material online, Table S4). These observations further support the notion that LOF-SCN5A mutations are phenotypically malignant and associated with LAE, while the reduction in peak  $I_{Na}$  density serves as the principal predictor of BrS disease risk. Further functional evaluations and larger scale clinical studies involving more SCN5A-positive cases are warranted to prove this hypothesis.

**Table 2** Lethal arrhythmic events in Brugada syndrome cohort-I subgroups classified by different SCN5A functional properties

Subgroups	Patient characteristics	Patient number	LAE during follow-up (%)	Annual event rate (%/year)	Estimated mean LAE-free period (months, mean $\pm$ SE)
All BrS	All BrS patients	415	62 (14.9)	2.5	206.9 $\pm$ 6.0
SCN5A(-)	Rare SCN5A variants non-carriers	355	49 (13.8)	2.2	210.8 $\pm$ 6.0
SCN5A(+)	Rare SCN5A variant carriers	60	13 (21.7)	5.1	136.6 $\pm$ 12.9
LOF	LOF-SCN5A mutation carriers	45	13 (28.9)	7.9	94.5 $\pm$ 10.7
Non-LOF	Non-LOF SCN5A variant carriers	15	0 (0)	0	NA
Non-LOF +SCN5A(-)	Patients without LOF-SCN5A mutations	370	49 (13.2)	2.1	208.8 $\pm$ 5.9

BrS, Brugada syndrome; LAE, lethal arrhythmic event; LOF, loss of function; SE, standard error; NA, not available.

**Table 3** Univariate analysis of lethal arrhythmic event during the follow-up of Brugada syndrome cohort-I

Variables	Hazard ratio (95% CI)	P-value
History of aborted cardiac arrest	6.58 (3.94–10.97)	5.3E-13
LOF-SCN5A mutations	3.18 (1.72–5.90)	2.4E-04
<i>In silico</i> -predicted rare SCN5A variants	2.08 (1.12–3.84)	0.02
History of syncope (without aborted cardiac arrest)	2.07 (0.92–4.69)	0.08
Male	1.43 (0.20–10.34)	0.72
VT/VF by programmed electrical stimulation	1.72 (0.98–3.02)	0.06
Family history of sudden cardiac death <sup>a</sup>	1.11 (0.56–2.18)	0.77
Documented atrial fibrillation	1.83 (1.02–3.27)	0.043
Late potential positive	1.50 (0.75–2.97)	0.25
Spontaneous type-I ST elevation	1.34 (0.74–2.44)	0.33
QRS ( $V_5$ ) $\geq$ 120 ms	2.57 (1.34–4.93)	0.005
P (II) $\geq$ 120 ms	2.71 (1.53–4.80)	0.001

VF, ventricular fibrillation; VT, ventricular tachycardia; CI, confidence interval.

<sup>a</sup>Prevalence at  $\leq$ 45 years old.

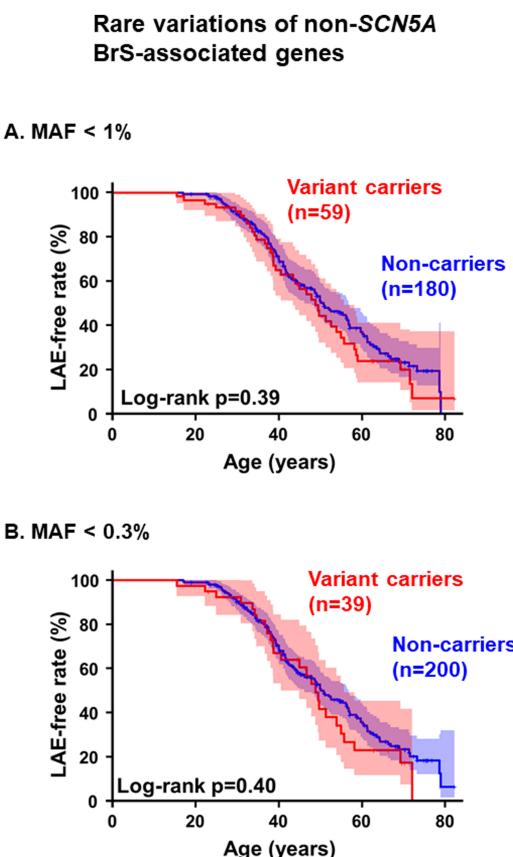
**Table 4** Multivariate analysis of lethal arrhythmic event during the follow-up of Brugada syndrome cohort-I

Variables	LOF-SCN5A mutations		<i>In silico</i> -predicted rare SCN5A variants	
	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
History of aborted cardiac arrest	6.31 (3.69–10.80)	1.7E-11	6.46 (3.77–11.09)	1.2E-11
SCN5A status	2.89 (1.50–5.57)	0.002	2.08 (1.10–3.95)	0.025
QRS ( $V_5$ ) $\geq$ 120 ms	1.11 (0.55–2.25)	0.77	1.25 (0.63–2.49)	0.53
Documented atrial fibrillation	1.02 (0.56–1.87)	0.94	0.98 (0.54–1.80)	0.95

Although >20 non-SCN5A-associated genes have been recognized in BrS, precise interpretation of the pathogenicity of rare variations of these genes is often challenging. Using rare variant burden analysis of BrS-associated genes, Le Scouarnec et al.<sup>15</sup> identified a significant enrichment of SCN5A coding variants only in BrS cases and not in controls, but not those of other BrS-associated genes. Using an evidence-based ClinGen approach, Hosseini et al.<sup>14</sup> concluded that SCN5A is the only gene classified with definitive evidence of disease causality in BrS. Herein, we used whole-exome sequencing in a larger

cohort of BrS patients lacking SCN5A mutations and demonstrated that the rare coding variations of non-SCN5A BrS-associated genes were neither enriched in BrS (Supplementary material online, Figure S6), nor modified the long-term prognosis of BrS patients (Figure 5). Our data further support the notion that LOF-SCN5A mutations, but not rare coding variants of other BrS-susceptible genes, are the genetic burden of LAE in BrS.

The absence of LOF-SCN5A mutations in a given patient with BrS does not necessarily suggest a benign prognosis since disease



**Figure 5** Kaplan-Meier analysis of lifetime lethal arrhythmic event-free survival in Brugada syndrome cohort-II with or without rare variants of Brugada syndrome-associated genes. Lethal arrhythmic event-free survival of Brugada syndrome cohort-II probands were comparable regardless of the presence of rare coding variants of 22 non-SCN5A Brugada syndrome-associated genes with two different minor-allele frequencies (MAF) (A: <1%, B: <0.3%).

presentation is affected by several factors, including age, sex, common single-nucleotide polymorphisms (SNPs) near the SCN5A/SCN10A/HEY2 genes,<sup>13</sup> and structural abnormalities including fibrosis and inflammation.<sup>12</sup> Considering that most (~80%) patients with BrS are mutation negative, it is speculated that the genetic risk of sudden death is also determined by both monogenic factors (rare LOF-SCN5A mutations) and polygenic factors (unidentified common variants) (*Graphical Abstract*). Although SNPs associated with sudden death or lethal arrhythmia have not been elucidated in BrS, it is possible that the polygenic contribution of BrS-associated common SNPs in SCN5A-negative BrS may be equivalent to or even greater than in SCN5A-positive BrS.<sup>20</sup>

## Study limitations

Patients in this study were exclusively of Japanese descent and limited in number; therefore, our study should be replicated using larger cohorts of different ethnicities. Electrophysiological properties of the variants were analysed based on heterologous expression; however,

some SCN5A variants might exhibit different properties in HEK293T cells as compared with those in cardiomyocytes or *in vivo*.<sup>21</sup>

## Conclusions

*In vitro* functional validation is a key method for classifying the pathogenicity of SCN5A VUSs. Functionally-validated LOF-SCN5A mutations contribute to the genetic burden of sudden death in BrS. Integrating the genetic information of LOF-SCN5A mutations with other rare or polygenic common risk variations, which are currently unknown, may help to develop a new personalized risk stratification paradigm for the complex oligogenic disease, BrS.

## Supplementary material

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

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## Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

**Conflict of interest:** All authors have submitted the ICMJE form for disclosure of potential conflicts of interest. H.M. reports an endowed chair from Japan Medtronic during the conduct of the study. A.N. reports grants from Medtronic and DVx and personal fees from Abbott, Johnson and Johnson, Daiichi-Sankyo, Bayer, and Boehringer Ingelheim, outside the submitted work. M.T. reports personal fees from Daiichi-Sankyo, Bayer Japan, Bristol-Myers Squibb, Boehringer Ingelheim, Japan Lifeline, Biotronik Japan, Abbott Medical Japan, and Medtronic Japan outside the submitted work. The other authors have no conflict of interest to declare.

## References

- Brugada J, Campuzano O, Arbelo E, Sarquella-Brugada G, Brugada R. Present status of Brugada syndrome: JACC state-of-the-art review. *J Am Coll Cardiol* 2018; **72**:1046–1059.
- Priore SG, Blomström-Lundqvist C, Mazzanti A, Blom N, Borggrefe M, Camm J, Elliott PM, Fitzsimons D, Hatala R, Hindricks G, Kirchhof P, Kjeldsen K, Kuck KH, Hernandez-Madrid A, Nikolaou N, Norekval TM, Spaalding C, Van Veldhuisen DJ. 2015 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death. *Eur Heart J* 2015; **36**:2793–2867.
- Probst V, Veltmann C, Eckardt L, Meregalli PG, Gaita F, Tan HL, Babuty D, Sacher F, Giustetto C, Schulze-Bahr E, Borggrefe M, Haissaguerre M, Mabo P, Le Marec H, Wolpert C, Wilde AA. Long-term prognosis of patients diagnosed with Brugada syndrome: results from the FINGER Brugada Syndrome Registry. *Circulation* 2010; **121**:635–643.
- Calo L, Giustetto C, Martino A, Sciarra L, Cerrato N, Marziali M, Rauzino J, Carlino G, de Ruvo E, Guerra F, Rebecchi M, Lanzillo C, Anselmino M, Castro A, Turreni F, Penco M, Volpe M, Capucci A, Gaita F. A new electrocardiographic

- marker of sudden death in Brugada syndrome: the S-wave in lead I. *J Am Coll Cardiol* 2016; **67**:1427–1440.
5. Yamagata K, Horie M, Aiba T, Ogawa S, Aizawa Y, Ohe T, Yamagishi M, Makita N, Sakurada H, Tanaka T, Shimizu A, Hagiwara N, Kishi R, Nakano Y, Takagi M, Makiyama T, Ohno S, Fukuda K, Watanabe H, Morita H, Hayashi K, Kusano K, Kamakura S, Yasuda S, Ogawa H, Miyamoto Y, Kapplinger JD, Ackerman MJ, Shimizu W. Genotype–phenotype correlation of SCN5A mutation for the clinical and electrocardiographic characteristics of probands with Brugada syndrome: a Japanese Multicenter Registry. *Circulation* 2017; **135**:2255–2270.
  6. Sommariva E, Pappone C, Martinelli Boneschi F, Di Resta C, Rosaria Carbone M, Salvi E, Vergara P, Sala S, Cusi D, Ferrari M, Benedetti S. Genetics can contribute to the prognosis of Brugada syndrome: a pilot model for risk stratification. *Eur J Hum Genet* 2013; **21**:911–917.
  7. Milman A, Andorin A, Postema PG, Gourraud JB, Sacher F, Mabo P, Kim SH, Maeda S, Takahashi Y, Kamakura T, Aiba T, Conte G, Juang JJM, Leshem E, Michowitz Y, Fogelman R, Hochstadt A, Mizusawa Y, Giustetto C, Arbelo E, Huang Z, Corrado D, Delise P, Allocca G, Takagi M, Wijeyeratne YD, Mazzanti A, Brugada R, Casado-Arroyo R, Champagne J, Calo L, Sarquella-Brugada G, Jespersen CH, Tfelt-Hansen J, Veltmann C, Priori SG, Behr ER, Yan GX, Brugada J, Gaia F, Wilde AAM, Brugada P, Kusano KF, Hirao K, Nam GB, Probst V, Belhassen B. Ethnic differences in patients with Brugada syndrome and arrhythmic events: new insights from Survey on Arrhythmic Events in Brugada syndrome. *Heart Rhythm* 2019; **16**:1468–1474.
  8. Kapplinger JD, Giudicessi JR, Ye D, Tester DJ, Callis TE, Valdivia CR, Makieliski JC, Wilde AA, Ackerman MJ. Enhanced classification of Brugada syndrome-associated and long-QT syndrome-associated genetic variants in the SCN5A-encoded Na(v)1.5 cardiac sodium channel. *Circ Cardiovasc Genet* 2015; **8**:582–595.
  9. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL; on behalf of the ACMG Laboratory Quality Assurance Committee. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet Med* 2015; **17**:405–424.
  10. Meregalli PG, Tanck MW, Probst V, Koopmann TT, Tanck MW, Bhuiyan ZA, Sacher F, Kyndt F, Schott JJ, Albusisson J, Mabo P, Bezzina CR, Le Marec H, Wilde AA. Type of SCN5A mutation determines clinical severity and degree of conduction slowing in loss-of-function sodium channelopathies. *Heart Rhythm* 2009; **6**:341–348.
  11. Glazer AM, Wada Y, Li B, Muhammad A, Kalash OR, O'Neill MJ, Shields T, Hall L, Short L, Blair MA, Kroncke BM, Capra JA, Roden DM. High-throughput reclassification of SCN5A variants. *Am J Hum Genet* 2020; **107**:111–123.
  12. Nademanee K, Raju H, de Noronha SV, Papadakis M, Robinson L, Rotherapy S, Makita N, Kowase S, Boonmee N, Vitayakritsirikul V, Ratanarapee S, Sharma S, van der Wal AC, Christiansen M, Tan HL, Wilde AA, Nogami A, Sheppard MN, Veerakul G, Behr ER. Fibrosis, connexin-43, and conduction abnormalities in the Brugada syndrome. *J Am Coll Cardiol* 2015; **66**:1976–1986.
  13. Bezzina CR, Barc J, Mizusawa Y, Remme CA, Gourraud JB, Simonet F, Verkerk AO, Schwartz PJ, Crotti L, Dagradi F, Guicheney P, Fressart V, Leenhardt A, Antzelevitch C, Bartkowiak S, Borggrefe M, Schimpf R, Schulze-Bahr E, Zumhagen S, Behr ER, Bastiaenen R, Tfelt-Hansen J, Olesen MS, Kaab S, Beckmann BM, Weeke P, Watanabe H, Endo N, Minamino T, Horie M, Ohno S, Hasegawa K, Makita N, Nogami A, Shimizu W, Aiba T, Froguel P, Balkau B, Lantieri O, Torchio M, Wiese C, Weber D, Wolswinkel R, Coronel R, Boukens BJ, Bezieau S, Charpentier E, Chatel S, Despres A, Gros F, Kyndt F, Lecointe S, Lindenbaum P, Portero V, Violleau J, Gessler M, Tan HL, Roden DM, Christoffels VM, Le Marec H, Wilde AA, Probst V, Schott JJ, Dina C, Redon R. Common variants at SCN5A-SCN10A and HEY2 are associated with Brugada syndrome, a rare disease with high risk of sudden cardiac death. *Nat Genet* 2013; **45**:1044–1049.
  14. Hosseini SM, Kim R, Udupa S, Costain G, Jobling R, Liston E, Jamal SM, Szybowska M, Morel CF, Bowdin S, Garcia J, Care M, Sturm AC, Novelli V, Ackerman MJ, Ware JS, Hershberger RE, Wilde AAM, Gollobo MH; National Institutes of Health Clinical Genome Resource C. Reappraisal of reported genes for sudden arrhythmic death: evidence-based evaluation of gene validity for Brugada syndrome. *Circulation* 2018; **138**:1195–1205.
  15. Le Scouarnec S, Karakachoff M, Gourraud JB, Lindenbaum P, Bonnau S, Portero V, Duboscq-Bidot L, Daumy X, Simonet F, Teusan R, Baron E, Violleau J, Persyn E, Bellanger L, Barc J, Chatel S, Martins R, Mabo P, Sacher F, Haissaguerre M, Kyndt F, Schmitt S, Bezieau S, Le Marec H, Dina C, Schott JJ, Probst V, Redon R. Testing the burden of rare variation in arrhythmia-susceptibility genes provides new insights into molecular diagnosis for Brugada syndrome. *Hum Mol Genet* 2015; **24**:2757–2763.
  16. Priori SG, Wilde AA, Horie M, Cho Y, Behr ER, Berul C, Blom N, Brugada J, Chiang CE, Huikuri H, Kannankeril P, Krahn A, Leenhardt A, Moss A, Schwartz PJ, Shimizu W, Tomaselli G, Tracy C. HRS/EHRA/APHRS expert consensus statement on the diagnosis and management of patients with inherited primary arrhythmia syndromes: document endorsed by HRS, EHRA, and APHRS in May 2013 and by ACCF, AHA, PACES, and AEPC in June 2013. *Heart Rhythm* 2013; **10**:1932–1963.
  17. ClinVar. <https://www.ncbi.nlm.nih.gov/clinvar/>. Date accessed 16 May 2020.
  18. Kroncke BM, Glazer AM, Smith DK, Blume JD, Roden DM. SCN5A (NaV1.5) variant functional perturbation and clinical presentation: variants of a certain significance. *Circ Genom Precis Med* 2018; **11**:e002095.
  19. Pearman CM, Denham NC, Mills RW, Ding WY, Modi SS, Hall MCS, Todd DM, Mahida S. Relationship between sodium channel function and clinical phenotype in SCN5A variants associated with Brugada syndrome. *Hum Mutat* 2020; **41**:2195–2204.
  20. Wijeyeratne YD, Tanck MW, Mizusawa Y, Batchvarov V, Barc J, Crotti L, Bos JM, Tester DJ, Muir A, Veltmann C, Ohno S, Page SP, Galvin J, Tadros R, Muggenthaler M, Raju H, Denjoy I, Schott JJ, Gourraud JB, Skoric-Milosavljevic D, Nannenberg EA, Redon R, Papadakis M, Kyndt F, Dagradi F, Castelletti S, Torchio M, Meitinger T, Lichtner P, Ishikawa T, Wilde AAM, Takahashi K, Sharma S, Roden DM, Borggrefe MM, McKeown PP, Shimizu W, Horie M, Makita N, Aiba T, Ackerman MJ, Schwartz PJ, Probst V, Bezzina CR, Behr ER. SCN5A mutation type and a genetic risk score associate variably with Brugada syndrome phenotype in SCN5A families. *Circ Genom Precis Med* 2020; **13**:e002911.
  21. Watanabe H, Yang T, Stroud DM, Lowe JS, Harris L, Atack TC, Wang DW, Hipkens SB, Leake B, Hall L, Kupershmidt S, Chopra N, Magnuson MA, Tanabe N, Knollmann BC, George AL Jr., Roden DM. Striking *In vivo* phenotype of a disease-associated human SCN5A mutation producing minimal changes *In vitro*. *Circulation* 2011; **124**:1001–1011.

## Corrigendum

### Corrigendum to: Pregnancy-associated arterial dissections: a Nationwide cohort study

*Eur Heart J* 2020; doi:10.1093/eurheartj/ehaa497

In the original publication of this article, an annotated version of the supplementary data file was erroneously published, and a clean Supplementary Data file has now replaced this online. Within the new Supplementary Data file, some figures have also been removed from tables to avoid patient identification.

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