Role of *CACNA1C* in Brugada syndrome: Prevalence and phenotype of probands referred for genetic testing

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BACKGROUND Evidence for the role of the CACNA1C gene, which encodes for the α-subunit of the cardiac L-type calcium channel CaV1.2, as a cause of the BrS3 variant of Brugada syndrome (BrS) is contradictory.

OBJECTIVE The purpose of this study was to define in a large BrS cohort the yield of molecular screening and to test whether appropriate patient selection could improve clinical utility.

METHODS A total of 709 patients were included in this study. BrS probands (n = 563, consecutively referred) underwent *CACNA1C* sequencing. Two matched cohorts where defined: discovery cohort (n = 200) and confirmation cohort (n = 363). In addition, the clinical phenotypes of a matched *SCN5A*-positive BrS cohort (n = 146) were included for comparative genotype-phenotype correlation.

RESULTS In the discovery cohort, we identified 11 different rare variants in 9 patients; 10 of the variants (5%) were considered potentially causative based on their frequency in the general population. However, American College of Medical Genetics criteria were unable to classify the majority (80%) of them, which eventually were labeled as variants of unknown significance (VUS). Functional studies revealed a loss of function for 9 variants, pointing to a prevalence of *CACNA1C* causative variants in 4% of the discovery cohort. Genotype-phenotype correlation showed that pathogenic variants are significantly more frequent in patients with shorter QTc (12.9% vs 2.2% in patients with QTc <390 ms).

CONCLUSION *CACNA1C* is an infrequent but definitive cause of BrS typically associated with short QT. Functional studies are highly relevant to improve variant interpretation.

KEYWORDS American College of Medical Genetics/Association for Molecular Pathology guidelines; Brugada syndrome; CaV1.2 calcium channel; Genetic testing; Genetic variants; Variant interpretation

(Heart Rhythm 2022;■:1–9)

Introduction

Genetic testing of Brugada syndrome (BrS) is far from being a settled field. Although 23 causative genes having been linked to the disease (Supplemental Table 1), mutations are found in a minority (~25%–30%) of subjects with a clinical diagnosis.[1] The *SCN5A* gene is the most prevalent and is the only gene confirmed by definitive evidence to be associated with BrS, while the clinical utility of systematic screening of the other genes remains under scrutiny due to the lack of information on prevalence and function often leading to an inconclusive interpretation of laboratory findings. Next-generation sequencing has opened a "veritable floodgate of biological data of unknown clinical significance"[2] to a point such that some investigators have suggested that, in the absence of functional data, the interpretation of variants is almost impossible.[3]

The second most frequent gene reported in association with BrS is *CACNA1C*, which encodes for the α-subunit of the voltage-gated calcium channel (locus BrS3). Initial reports suggested that it accounts for a clinically relevant percentage of BrS variants (up to 12%),[1,2,4] but this has not been confirmed by other investigators.[3,5] Systematic evidence-based evaluation of published genetic variants showed that approximately half of the *CACNA1C* variants cannot be conclusively labeled as "definitive" disease-causing genes.[5] Despite these limitations, it is still generally agreed that *CACNA1C* analysis can be relevant for familial screening and follow-up management.

Funding Sources: The study was supported by the CARIPLO project (2008.2504) and by the Italian Ministry Ricerca Corrente Program. Disclosures: The authors have no conflicts of interest to disclose. **Address reprint requests and correspondence:** Dr Carlo Napolitano, Molecular Cardiology, ICS Maugeri, IRCCS, Via S. Maugeri 10, 27100 Pavia, Italy. E-mail address: carlo.napolitano@unipv.it.

  https://doi.org/10.1016/j.hrthm.2021.12.032

In this study, we aimed to define the prevalence of *CACNA1C* variants in a large series of BrS probands. We also aimed to test whether *CACNA1C*-positive patients have genotype-specific phenotypes that could improve the pretest probability of pathogenic variants or support the interpretation of genetic findings.

We designed the study to be informative for the typical BrS scenario in which genetic testing is initiated in a proband who comes to medical attention with no or scanty familial clinical data. This is a challenging and frequent presentation for cardiologists upon the initial contact with a BrS patient, given the incomplete penetrance and variable expressivity of the disease that make co-segregation hard to define.[6–8]

To reach our study goals, a stepwise approach was used. The identified variants initially were classified by integrating variable evidence readily available from a diagnostic laboratory. Typically, genetic testing was performed and the results returned to the physician. Functional studies then were performed to confirm the prediction of the pathogenic effect of the identified *CACNA1C* variants included in the discovery cohort. Lastly, we evaluated whether *CACNA1C* carriers showed a unique phenotype that would enable personalized genetic testing for these patients.

Methods

Study population

A total of 563 SCN5A-negative BrS probands were divided into 2 groups: discovery cohort (n = 200 consecutive subjects) and confirmation cohort (n = 363 patients). In addition, a matched *SCN5A*-positive BrS cohort (n = 146) was included for analysis of genotype-phenotype correlation. Overall, the study cohort included 709 patients with BrS diagnosed according to the 2013 Heart Rhythm Society/European Heart Rhythm Association/Asia Pacific Heart Rhythm Society guidelines.[9] QT was measured at enrollment electrocardiogram (ECG) at a stable heart rate between 55 and 75 bpm and validated blindly by 2 investigators. A third independent observer made a final validation of measures in cases with >20-ms interobserver difference. Cardiac events were defined as syncope, cardiac arrest, and appropriate implantable cardioverter-defibrillator shocks.[9]

Genetic screening and variant interpretation

Genetic analysis was performed by conventional Sanger sequencing (Applied Biosystems, Dreieich, Germany). In brief, the complete coding regions of *SCN5A* (NM_000335; LRG_289t2) and *CACNA1C* (NM_000719; LRG_334t1 and NM_001129827; LRG_334t2), including 8 alternative exons, were analyzed.

Variant interpretation was performed using the American College of Medical Genetics (ACMG)/for Molecular Pathology (AMP) guidelines using a disease-specific allele-frequency threshold adapted to ethnicity, disease prevalence, and penetrance.[10] In detail, we used the following criteria to ascertain evidence of pathogenicity: allele frequency ≤0.1% reported in gnomAD browser (v2.1.1 release) referring to European (non-Finnish) population and Uniprot database (uniprot.org) to identified variants located in critical functional domains.

In silico prediction tools Polyphen2, SIFT, and Splice Site Prediction by Neural Network were used to assess the *in silico* effect of missense variants and splicing alterations.

All patients gave formal consent to genetic testing and release of their clinical data. The protocol was approved by the ICS Maugeri ethical committee.

Cellular electrophysiology and immunofluorescence

Site-directed mutagenesis was performed on full-length human transcript variant 17 *CACNA1C* (NM_001129843) cloned in pCMV6-XL4 (Origene Technologies, Rockville, MD) using the QuickChange II XL kit (Agilent Technologies, Santa Clara, CA). Transfection of HEK293 cells was performed using Effectene (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol. A complete description of the *in vitro* studies is provided in the Supplemental Materials.

Statistical analysis

The following groups were compared for QTc analysis: CACN1C-positive vs CACNA1C-negative vs SCN5A-positive. Study variables were tested for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Differences between groups were quantified with independent samples *t* test or 1-way analysis of variance with Bonferroni correction for multiple comparisons. The χ^2 test with Fisher exact test was applied for categorical variables with Bonferroni correction if required by multiple comparisons. Receiver operator characteristic (ROC) analysis and the identification of optimal cutpoints for sensitivity and specificity were performed using a specific R package.[11] The matched *SCN5A* cohort was drawn from our clinical database using age, gender, and incidence of spontaneous type 1 at baseline ECG as matching variables. QTc values are expressed as mean ± SD. Standard error was used for electrophysiological data. Except for ROC analysis, data were analyzed with the SPSS statistical package Version 23 (SPSS, Chicago, IL).

The study was approved by the ICS Maugeri Ethical Committee and was compliant with the Helsinki Declaration as revised in 2013.

Results

Identification of *CACNA1C* genetic variants

In the discovery study cohort (n = 200) (Supplemental Table 2), we identified 11 putative different pathogenic variants in 9 patients (6%), including 9 missense, 1 in-frame deletion, and 1 splicing variant. Five variants were novel to this report; the others were previously reported (Table 1). Two probands carried 2 variants (p.Q428E-p.N1255S, p.R1880Q-p.R1973Q).

Most of the missense variants (45%) occurred in the C-terminal region (p.A1648T, p.A1717G, p.R1880Q, p.R1973Q,

Table 1 Assessment of CACNA1C variants identified in the discovery cohort

Reference no.	Variant	Location	MAF (%)*	Polyphen2	SIFT	Evidence satisfied	First tier annotation	I_{ca} current	Final variant classification
22	p.T320M	DI/S5-S6	0.006	Damaging	Damaging	PM2, PP3	VUS	↓ 73%	Likely pathogenic
Novel	p.Q428E†	AID	NA	Damaging	Damaging	PM1, PM2, PP3	VUS	↓ 52%	Likely pathogenic
Novel	p.E850D	PEST	NA	Damaging	Tolerant	PM1, PM2	VUS	No current	Likely pathogenic
14	p.E850del‡	PEST	NA	NA	NA	PM1, PM2, PS3	Likely pathogenic	↓ 54%	Likely pathogenic
Novel	p.N1255S†	DIV/S1	NA	Damaging	Damaging	PM2, PP3	VUS	↓ 80%	Likely pathogenic
15	p.A1648T‡	C-terminal	0.01	Benign	Tolerant	PM2, BP4	VUS	↓ 77%	Likely pathogenic
23	p.A1717G‡	C-terminal	0.06	Benign	Tolerant	PM2, BP4	VUS	No current	Likely pathogenic
2	p.R1880Q†	C-terminal	0.02	Benign	Tolerant	PM2, BP4	VUS	No current	Likely pathogenic
15	p.R1973Q†	C-terminal	0.16	Benign	Tolerant	BS1, BP4, BS3	Likely benign	NA	Benign
Novel	p.G2084E	C-terminal	NA	Damaging	Tolerant	PM2	VUS	↓ 60%	Likely pathogenic
Novel	c.1482-3 delT	NA	NA	NA	NA	PM2, PP3	VUS	NA	VUS

NA = not available.
*MAF (minor allele frequency) refers to data reported in gnomAD population European (non-Finnish) (v2.1 release).
†Identified in the same probands as p.Q428E + p.N1255S.
‡Previously functional evaluated.

p.G2084E); only 3 (p.Q428E, p.E850D, p.E850del) were located in the interdomain loops, 1 in the extracellular linker between S5-S6 of DI (p.T320M), and 1 (p.N1255S) in the S1 transmembrane domain of DIV (Figure 1). Three variants occurred in known relevant protein domains: p.Q428E modified the first amino acid of the α_1-subunit interaction domain,[12] which is responsible for binding with the Cavβ_2 subunit and variants; and p.E850del and p.E850D located in the PEST region, a sequence motif[13] rich with the amino acids (proline [P], glutamic acid [E], serine [S], threonine [T]), which provides a signal for rapid protein degradation and LTCC protein stability. All variants reported were identified in transcript variant 18 of the gene (NM_000719; LRG334_t1) (Table 1).

Variant interpretation

At the first tier of variant interpretation, we applied only evidence usually available from diagnostic laboratories: clinical diagnosis, minor allele frequency, data derived from the medical literature, and functional prediction by *in silico* tools. Ten of 11 variants presented minor allele frequency $\leq$0.1% (91%) reported in the European (non-Finnish) population (Table 1).

In silico prediction of missense variants was concordant for 7 variants: 3 classified as pathogenic (p.T320M, p.Q428E, p.N1255S) and 4 as benign (p.A1648T, p.A1717G, p.R1880Q, p.R1973Q), respectively. In the remaining cases, there was a discordant prediction. Splicing prediction confirmed the pathogenicity for variant c.1482-3 delT. Functional evidence from published studies was available for 4 variants: p.E850del, p.A1648T, and p.A1717G, p.R1973Q.[2,14,15] However, due to inconsistent results, we applied functional evidence (ACMG-PS3 rule) only for the 2 variants p.E850del and p.R1973Q.

At this step, due to lack of sufficient evidence for definitive classification, the majority of the variants (81%) were classified as variants of unknown significance (VUS). This reassessment was consistent with that reported in the ClinVar database when available. Only 2 variants, p.E850del and p.R1973Q, were classified as likely pathogenic and likely benign, respectively (Table 1).

Functional studies on CACNA1C variants

We then aimed to gather experimental support to determine functional evidence for the missense variants of the discovery cohort for which functional assessment was unavailable or nonconclusive, including the one classified as likely benign (p.R1973Q) from our first annotation. Individual mutants p.T320M, p.Q428E, p.E850D, p.N1255S, p.A1648T, p.A1717G, p.R1880Q, p.R1973Q, and p.G2084E were expressed in HEK293 cells in the presence of Cavβ_2 and Cav$\alpha_2\delta_1$ subunits. We compared the current-voltage relationship between wild-type (WT) and each mutation, and a series of depolarization pulses was applied in 10-mV increments from a holding potential of –80 mV. The results of these experiments are given in Figure 2 and Supplemental Table 3. To determine whether the complete loss of current in 4 variants

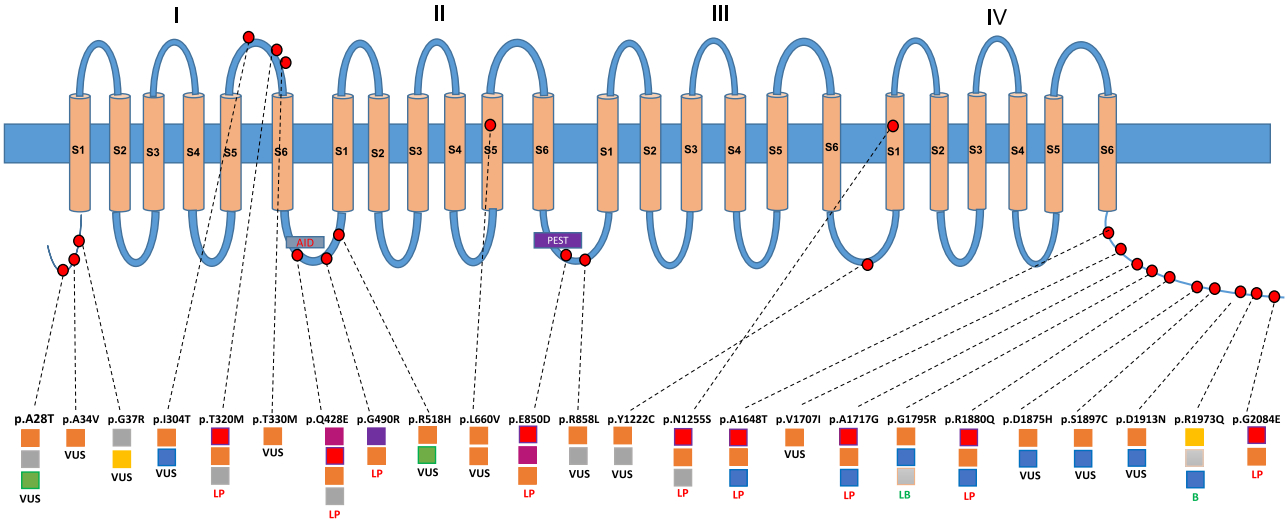

Figure 1 CACNA1C protein domain structure. Missense variants identified in both cohorts and their variant interpretation. *Red circles* represent the missense variants identified in this study. *Squares* represent the different American College of Medical Genetics criteria applied for variant interpretation: *red* = PS3 (functional assays); *orange* = PM2 (population frequency); *gray* = PP3 (*in silico* prediction); *violet* = PM1 (located in functional domain); *green* = BP5 (alternate molecular basis for disease); *yellow* = BS1 (population frequency); *blue* = BP4 (*in silico* prediction); *pink* = BS3 (no damaging effect). B = benign; LP = likely pathogenic; VUS = variant of uncertain significance.

(p.E850D, p. V1707I, p.A1717G, p.R1880Q) was caused by trafficking defects, we further investigated these genetic alterations by confocal microscopy. Data indicated a clearly different subcellular distribution between WT and mutants (Figure 3). In particular, the WT protein was predominantly localized along the plasma membrane, whereas p.E850D, p.V1707I, p.A1717G, and p.R1880Q mutants had a fluorescence pattern exclusively localized to cytoplasmic and perinuclear regions. These data suggest that intracellular retention of the mutant protein is a frequent mechanism of BrS-related *CACNA1C* defects.

By combining *in vitro* cellular electrophysiological data collected in this study and the genetic and functional evidence previously evaluated, 9 variants were definitively classified as likely pathogenic, 1 as VUS, and 1 as benign, confirming a prevalence of 4.0% (8/200 probands). These results support the concept that for diseases with incomplete penetrance and a high incidence of sporadic cases, only strong clinical evidence and *in vitro* functional assays can reap the ability VUS reclassification.[16]

Furthermore, the assumption that a variant cannot be more common in the population than the disease it causes can be not always true if a variant shows incomplete penetrance due to the presence of strong modifiers.[17] In this case, frequency assessment using an adaptation of the PM2 criterion had great value in discriminating variants that later were confirmed as pathogenic by in *vitro* studies.

Confirmation cohort

At a second step, we screened 363 BrS patients and identified 23 different genetic variants in 27 patients, including 5 splice-altering variants, 17 missense, and 1 in-frame deletion. Two missense (p.A1717G and p.R1973Q) and 1 in-frame indel (p.E850del) were previously identified in the discovery cohort. Five missense (p.A28T, p.G37R, p.G490R, p.R518H, p.G1795R) were also reported in other studies.[1,2,14,18] The remaining 15 variants were novel. More than 40% of the missense variants were located in C-terminal regions (p.V1707I, p.A1717G, p.G1795R, p.D1875H, p.S1897C, p.D1913N, p.R1973Q), 18% in N-terminal regions (p.A28T, p.A34V, p.G37R), and the remaining variants in interlinker regions (p.I304T, p.T330M, p.G490R, p.R518H, p.R858L, p.Y1222C), except for 1 variant (p.L660V) located in the transmembrane domain DII-S5 (Figure 1). Only 1 variant of this cohort was identified in an alternative transcript (NM_001129830). Variant interpretation for this cohort of patients is given in Table 2. For this group, variant interpretation was performed using the same adapted version of ACMG guidelines of the discovery cohort but with direct application of all evidence, genetic and experimental, that was available. As shown in Table 2, 4 variants (2.2%) were classified as likely pathogenic, 17 as VUS, and 2 as benign/likely benign (1 each).

The lack of strong evidence from functional studies and segregation data in this cohort supports a clear clinical interpretation for only 7 variants, confirming a molecular diagnosis in 5 patients (1.4%).

Genotype-phenotype correlation

Because *CACNA1C* variants in BrS are loss of function, we addressed whether a shorter QTc could be a hallmark of this genetic subtype of the disease. To test this hypothesis, we compared the QTc of CACNA1C-positive patients (pathogenic and likely pathogenic variants) with that of CACAN1C-negative patients with a matched cohort of SCN5A-positive BrS patients. This latter group was drawn

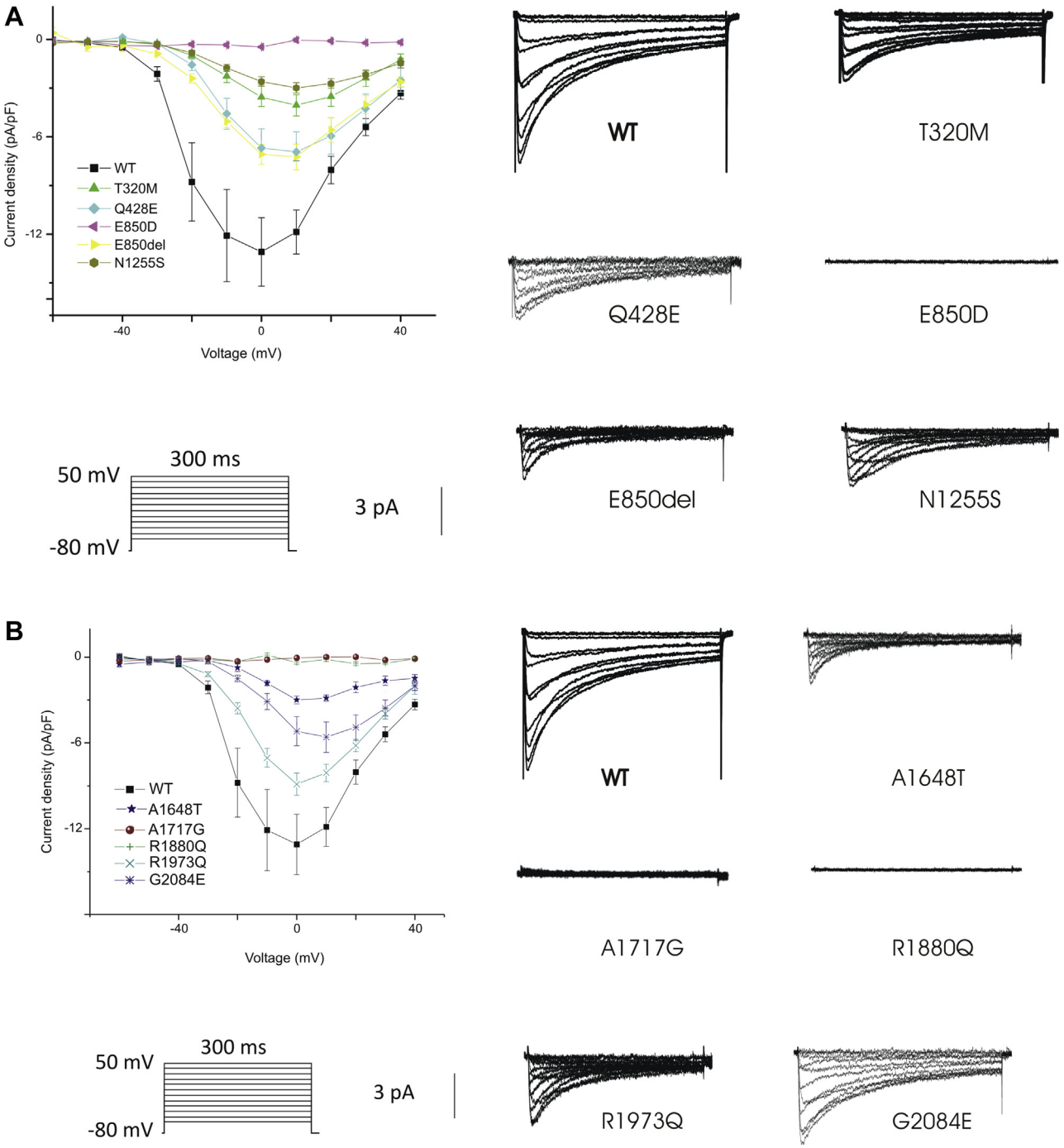

Figure 2 **A:** Representative whole-cell current traces of wild-type (WT) and mutant channels. Whole-cell current recording of WT, p.T320M, p.Q428E, p.E850D, p.E850del, and p.N1255S channels. Mutant CACNA1C channels reduced peak current compared to WT channel. **B:** Representative whole-cell current traces of WT and mutant channels. Whole-cell current recording of WT, p.A1648T, p.A1717G, p.R1880Q, p.R1973Q, and p.G2084E channels. Mutant CACNA1C channels reduced peak current compared to WT channel.

from our database using gender, the incidence of spontaneous type 1 ECG, and the incidence of cardiac events as matching categories. One hundred forty-six SCN5A mutation carriers were identified. In order to check for homogeneous QTc distribution in the overall study cohorts, we compared QTc in the discovery, confirmation, and SCN5A-positive groups. No significant QTc differences were found among the 3 cohorts (discovery: 399 ± 19 ms; confirmation 402 ± 16 ms; SCN5A 398 ± 26 ms; analysis of variance ANOVA $P = .064$).

However, when patients with confirmed pathogenic and likely pathogenic *CACNA1C* variants (N = 13) were compared with those without mutations (N = 511), a shorter QTc was clearly evident (400 ± 18 ms vs 371 ± 16 ms; $P = .000004$ for *CACNA1C* WT vs mutations carriers). Interestingly, genetic variants labeled as VUS had QTc similar to that of noncarriers (399 ± 18 ms).

The incidence of *CACNA1C* causative variants by QTc quartiles was shifted toward low QTc values: 12.9% (n =

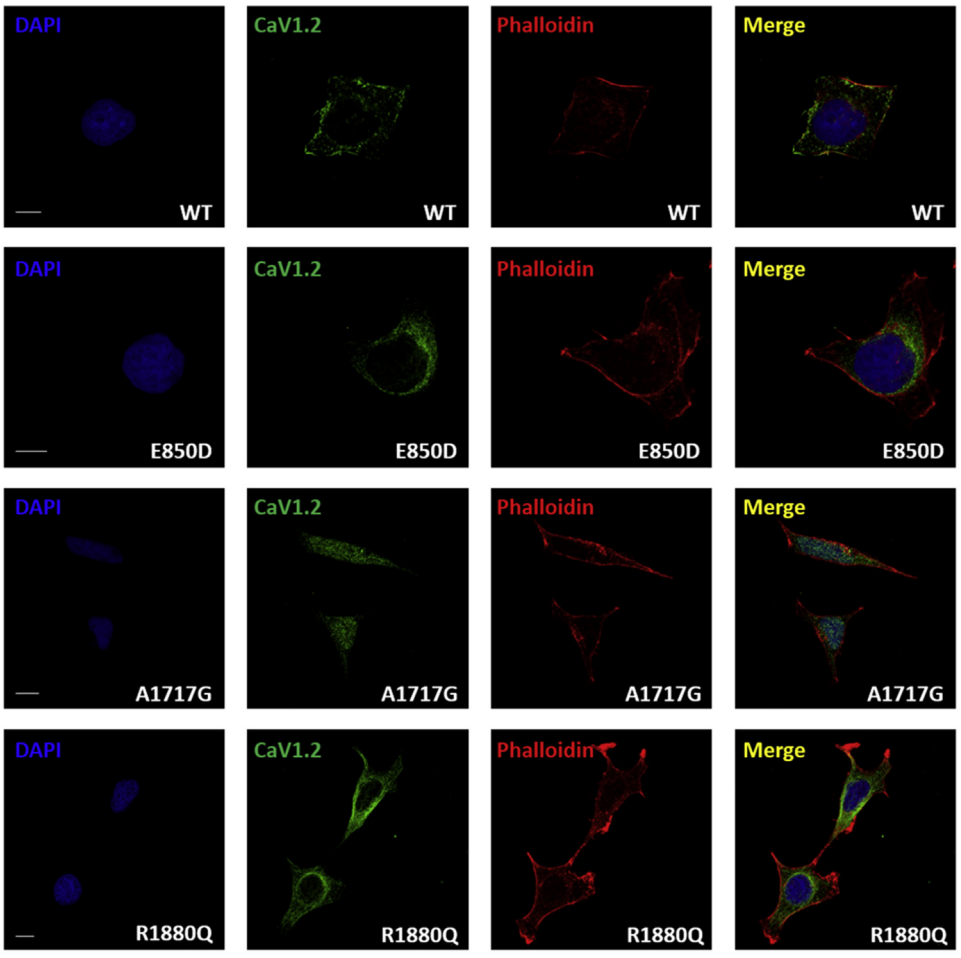

Figure 3 Loss of current caused by trafficking defects. Four variants causing loss of current (p.E850D, p.V1707I, p.A1717G, p.R1880Q) were studied by confocal microscopy to investigate the presence of trafficking defects. WT = wild-type.

14/108) in the group of patients with QTc in the lowest quartile (QTc <390 ms; χ^2 P <.000001).

ROC analysis showed an area under the curve of 0.91, suggesting a robust predictive accuracy of QTc in predicting the presence of a *CACNA1C* mutation (Figure 4A). The QTc cutoff value that better identified the carriers of *CACNA1C* mutations was 385 ms (Figure 4B), with sensitivity of 0.87 (95% confidence interval 0.60–0.98) and specificity of 0.85 (95% confidence interval 0.82–0.88). Therefore, causative *CACNA1C* BrS variants are associated with a shorter QT interval. No difference in the incidence of cardiac events was found among study cohorts (see Study limitations).

Discussion

Yield of *CACNA1C* genetic screening in BrS and variant annotation

Genetic testing in patients with BrS remains challenging due to genetic heterogeneity, the growing number of identified rare genes, and the overall low yield. Other than for the BrS1 locus (*SCN5A* gene), which has been consistently implicated in 15%–25% of cases, the yield of systematic screening of the remaining genes is poorly defined. Loss-of-function *CACNA1C* variants were associated with BrS3 variants of BrS. Previous reports of small cohorts have suggested the yield of genetic screening in BrS ranges from 0% to 5.5%.[2–4] A systematic evidence-based evaluation of published *CACNA1C* variants has questioned the clinical validity role of *CACNA1C* gene in BrS genetic testing.[5]

Here we report the results of systematic molecular screening of the entire *CACNA1C* open reading frame (including alternatively spliced exons) in the largest cohort of subjects analyzed for this genetic locus to date. Our data show that the incidence of disease-causing variants is overall very low (2.0% and 1.3% in the discovery and confirmation cohorts, respectively). *In vitro* functional characterization proved to be an important step in providing support to variant interpretation and has the potential to resolve VUS, thereby increasing the clinical utility of genetic testing.[16] However, the increasing widespread use of genetic testing by commercial laboratories and the current level of reimbursements from both public health care systems and private insurance companies do not leave a margin for systematic *in vitro* expression of all VUS. However, our results and those of other investigators highlight the need for a revision of these policies, at least for genetic testing of channelopathies for which *in vitro* expression is generally feasible with relatively low effort.

Table 2 Assessment of CACNA1C variants identified in the confirmation cohort

Ref.	Variant	Location	MAF (%)*	Polyphen2	SIFT	Evidence satisfied	I_{ca} current	Final variant classification
Novel	c.-3T>C	5′ UTR	0.003	NA	NA	PM2, PP3	NA	VUS
Novel	c.1218-3del	NA	NA	NA	NA	PM2	NA	VUS
Novel	c.3357-7-5 del	NA	0. 0002	NA	NA	PM2	NA	VUS
Novel	c.5589-1G>A	NA	0.007	NA	NA	PVS1, PM2	NA	Likely pathogenic
18	p.A28T†	N-terminal	0.005	Damaging	Damaging	PM2, PP3, BP5	↑> 60%	VUS
Novel	p.A34V	N-terminal	0.001	Benign	Tolerant	PM2	NA	VUS
2	p.G37R	N-terminal	0.5	Damaging	Damaging	BS1, PP3	NA	VUS
Novel	p.I304T	DI/S5-S6	0.09	Benign	Tolerant	PM2, BP4	NA	VUS
Novel	p.T330M	DI/S5-S6	0.002	Damaging	Tolerant	PM2	NA	VUS
1	p.G490R†	DI-DII	0. 02	Damaging	Tolerant	PM2, PS3	No current	Likely pathogenic
24	p.R518H‡	DI-DII	NA	Damaging	Tolerant	PM2, BP5	↓ 70%	VUS
Novel	p.L660V	DII/S5	NA	Damaging	Damaging	PM2, PP3	No	VUS
14	p.E850del†	PEST	NA	Damaging	Tolerant	PM1, PM2, PS3	↓ 54%	Likely pathogenic
Novel	p.R858L	DII-DIII	NA	Damaging	Damaging	PM2, PP3	NA	VUS
Novel	p.Y1222C	DIII-DIV	NA	Damaging	Damaging	PM2, PP3	NA	VUS
Novel	p.V1707I	C-terminal	0.03	Damaging	Tolerant	PM2	NA	VUS
This study	p.A1717G†	C-terminal	NA	Benign	Tolerant	PM2, PS3	No current	Likely pathogenic
15	p.G1795R†	C-terminal	0.01	Benign	Tolerant	PM2, BP4, BS3	No alteration	Likely benign
Novel	p.D1875H	C-terminal	0. 0008	Benign	Tolerant	PM2, BP4	NA	VUS
Novel	p.S1897C*	C-terminal	0. 003	Benign	Tolerant	PM2, BP4	NA	VUS
Novel	p.D1913N	C-terminal	NA	Benign	Tolerant	PM2, BP4	NA	VUS
15	p.R1973Q†	C-terminal	0.16	Benign	Tolerant	BS1, BP4, BS3	↓ 35%	Benign
Novel	p.A2039A	C-terminal	NA	NA	NA	PM2	no	VUS

NA = not available.

*MAF (minor allele frequency) refers to data reported in gnomAD population European (non-Finnish) (v2.1 released).

†Variants previously functionally characterized.

‡Previously suggested as a cause of long QT syndrome by studies. This mutation causes a 70% reduction of current density and an increase of window current. The final effects are difficult to predict. The patient had type 1 electrocardiogram and borderline QTc.

In case of lack of segregation data or incomplete penetrance, as in our case, functional studies had the capacity to reclassify an additional 3% of VUS as likely pathogenic. Of note, the prevalence of rare *CACNA1C* variants in the GnomAD database (average 238798 alleles) is 0.3%. This demonstrates an enrichment of *CACNA1C* variants in BrS of approximately 10 times based on our data, thus allowing us to conclude that this gene is associated with BrS, albeit with low prevalence.

Our results suggest that the interpretation of *CACNA1C* missense variants in BrS probands is very difficult in the absence of functional evidence and call for large-scale collaborative studies aimed at the systematic assessment of identified variants and, if not previously suggested, for BrS screening.

CACNA1C in BrS and phenotype

BrS-related *CACNA1C* variants typically are loss of function and lead to shorter repolarization. Therefore, it is mechanistically expected that carriers of causative variants of this gene will have a shorter QT interval. This behavior was suggested in the initial identification of BrS-associated *CACNA1C* variants.[1] Our data demonstrate that causative *CACNA1C* variants are associated with shorter QTc compared to that in *SCN5A* carriers and noncarriers. Of note, in the subgroup of BrS patients with QTc in the lowest quartile, the prevalence of *CACNA1C* variants was significantly higher (12%).

As *SCN5A* mutations are more frequent in the presence of conduction block,[19,20] the majority of *CACNA1C* variant carriers have QTc in the lower limb of the normal distribution. This now can be considered a typical feature of BrS3.

The incomplete penetrance[7] and the high incidence of sporadic cases[18] of BrS complicate the interpretation and clinical use of genetic testing. The annotation of genetic variants for clinical decision-making is a probabilistic procedure. Our data demonstrate that the yield of molecular screening of *CACNA1C* depends on precise phenotype definitions and the availability of functional characterization and, in a broader view, strengthen the concept that the clinical utility of genetic testing is highly correlated with the appropriate selection of patients.

Study limitations

Although we performed *in vitro* cellular expression and trafficking experiments, the complexity of modulatory mechanisms that may complicate the clinical expressivity of genetic variants is such that experimental support for clinical genotyping is arduous to achieve. Post-transcriptional regulation single-nucleotide polymorphisms and epigenetics components have not been analyzed. More specifically, the *CACNA1C* gene has a very complex genomic organization with multiple splicing variants that may modulate protein and current expression.[21] *In vitro* functional characterization has practical feasibility limitations that should be addressed, at least for genetic testing of channelopathies.

Subjects with a short QT interval tend to have impaired QT/RR adaptation. This was not addressed in this study

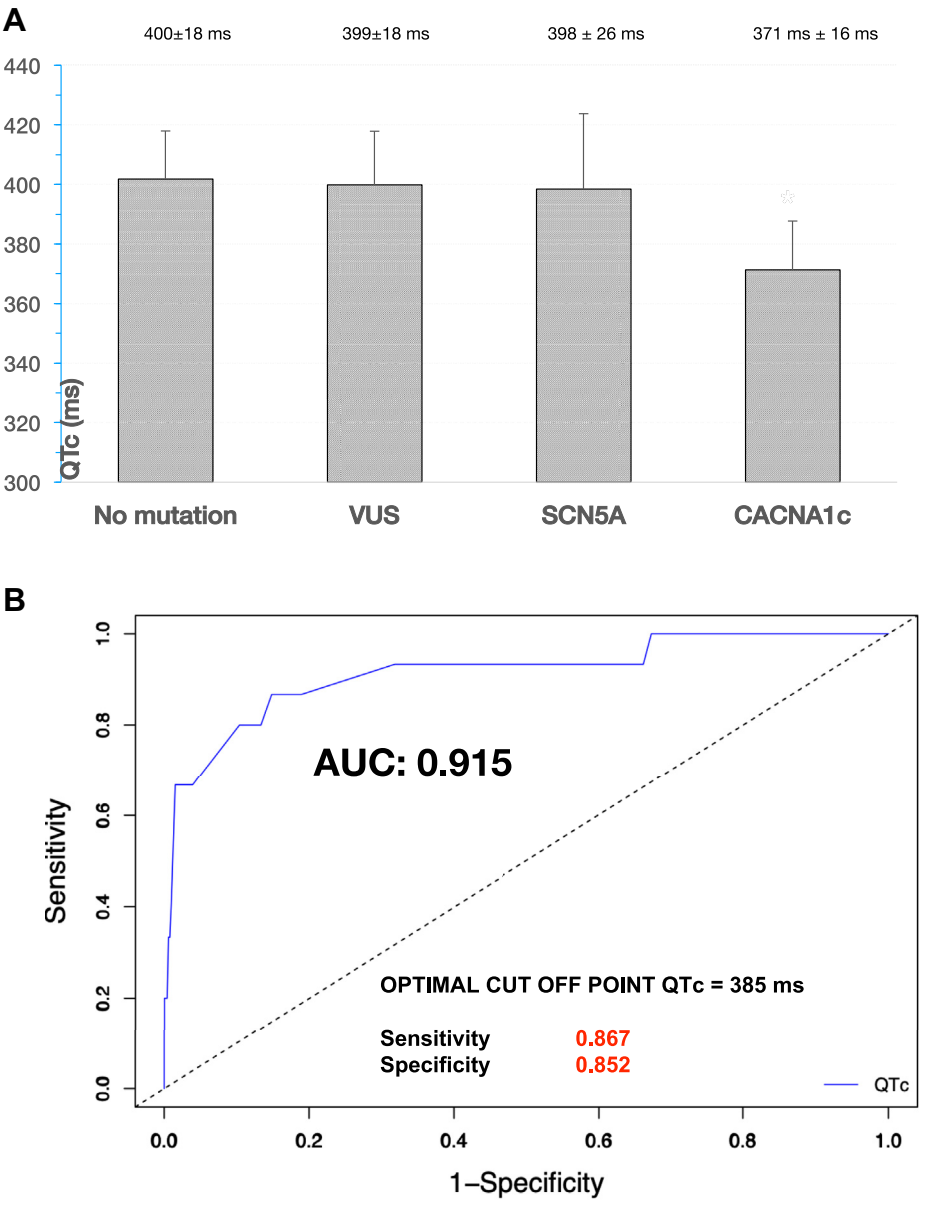

Figure 4 **A:** Genotype-phenotype correlation. QTc values in the study groups (see text for details). *P <.00001. **B:** Predictive accuracy of mutations. Receiver operator characteristic curves show the performance of QTc in identifying subjects with *CACAN1C* mutations. AUC = area under the curve; VUS = variant of unknown significance.

because Holter and exercise stress tests (to measure QT at low and high rates) were not available for all patients.

The endpoint of the study was addressing the yield in probands of genetic screening for BrS, which is the most frequent clinical condition in BrS, a disease with a relatively high percentage of sporadic cases. Family history of BrS or juvenile sudden cardiac death was present in 66% of the discovery cohort. However, we did not systematically analyze the patients with ajmaline/flecainide test, so this value can be underestimated. It is possible that co-segregation analysis could have contributed additional insights, but this seldom is performed in the daily clinical setting, especially outside of highly specialized centers. This limitation also applies to the analysis of arrhythmic events, which showed no association, but *post hoc* statistics showed that the study was underpowered for this endpoint, which was not originally planned in the study design

Conclusion

Despite the high prevalence of VUS and the need for rigorous variant interpretation, CACNA1c is a definite cause of BrS with a higher incidence among patients presenting a QT interval in the lower range of normal distribution. Translational knowledge at clinical, genetic and molecular/biophysical level is required for correct variant classification.

Acknowledgments

The authors would like to thank the Exome Aggregation Consortium and the groups that provided exome variant

data for comparison (20). We acknowledge the contribution of the European Reference Network–Guard Heart Network. We thank Elisa Tavazzani, PhD, for her expert support with cellular electrophysiology.

Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.hrthm.2021.12.032.

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