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A consistent arrhythmogenic trait in Brugada syndrome cellular phenotype

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Short title: Role of late sodium current in Brugada syndrome

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Dear Editor,

Brugada syndrome (BrS) is an inherited arrhythmic disease predisposing to sudden cardiac death (SCD), characterized by a typical electrocardiogram pattern that includes a J point elevation with a coved type ST segment.¹ BrS is a complex genetic disease in which ~20% of patients carry rare variants in *SCN5A* gene whereas the others remain genetically unresolved.² Despite this genetic complexity, we hypothesize that a common cellular phenotypic trait is at the root of this specific BrS ECG pattern. In this study, we identified a phenotype that is common to human induced pluripotent stem cell-derived ventricular cardiomyocytes (hiPSC-CMs) generated from six Brugada patients with different genetic backgrounds. Our results unmasked a cellular arrhythmogenic phenotype combining gene expression and electrical abnormalities, including an increase in late sodium current.

Six patients affected by type I BrS (BrS1-6; Figure S1; Table S1; Table S2) with a familial history of SCD or syncope were selected, among whom two carry *SCN5A* variants (marked with a ⁺ symbol). An additional individual, not affected by BrS (Non-BrS), carrying the same *SCN5A* variant as BrS2⁺, was also recruited, as well as four control (Ctrl) subjects. Somatic cells from all studied subjects were reprogrammed into hiPSC lines and differentiated into cardiomyocytes (Figure 1).

Transcriptional expression profiling identified 133 differentially expressed genes in BrS hiPSC-CMs (Figure 2A). Gene Set Enrichment Analyses showed that transcripts of transmembrane transporters and channels were significantly overrepresented (Figure 2B), including genes encoding sodium, calcium, and potassium channels (Figure 2C). High-throughput real-time RT-PCR,³ on 96 genes related to cardiac electrical function (Table S3) identified 13 differentially expressed genes in BrS, in comparison to Ctrl and Non-BrS hiPSC-CMs (Figure 2D). Importantly, the expression of *SCN5A*, the main BrS culprit gene identified to-date,⁴ remained unchanged, excluding *SCN5A* expression levels as a hallmark for BrS hiPSC-CM phenotype. Conversely, calcium and sodium transporters, playing important roles in membrane depolarization, were differentially expressed. Comparative analysis of hiPSC-CM electrophysiological functions investigated whether these modifications were a consistent trait of BrS phenotype at the cellular level.

Whereas decrease in sodium current is considered as the most frequently associated electrical alteration in BrS pathophysiology,^{5,6} protein expression of Na_v1.5, encoded by *SCN5A*, was decreased in only 2 BrS, and the Non-BrS lines (Figure 2E). Concordantly, reduction in I_{Na} density was detected in these same lines (Figure 2F-2H). This confirmed previous results, for BrS5⁺,⁷ and regarding BrS1⁺, which carries an *SCN5A* rare variant, the reduction was confirmed using conventional transfection in COS-7 cells of this variant (Figure S2). Furthermore, the steady-state activation and inactivation gating properties were not modified in BrS hiPSC-CMs (Figure S3A; Table S4). Therefore, I_{Na} reduction is not a common trait of BrS hiPSC-CMs and appears to be solely associated with the presence of variants affecting *SCN5A* expression or function.

Similarly, reduction in $I_{Ca,L}$ channel protein expression and current density were not a common trait of BrS hiPSC-CMs (Figure 2I-2L, Figure S3B; Table S4).

Global cellular electrophysiological phenotype was then evaluated with action potential (AP) recordings, but no AP basal parameters specifically segregated BrS hiPSC-CMs, and spontaneous beating frequencies did not differ between all cell lines (Figure S4). Noteworthy, ventricular-like AP analysis revealed an arrhythmic phenotype present mostly in BrS hiPSC-CMs, irrespective of their genetic background (Figure 3A). Early afterdepolarizations (EADs) were observed in 39% to 70% of all six BrS ventricular-like hiPSC-CMs versus only in 4% and 4.7% of Ctrl and Non-BrS hiPSC-CMs, respectively (Figure 3B, S5). Thereby, the high EAD occurrence in ventricular-like hiPSC-CMs was associated with the presence of a BrS phenotype in the investigated cell lines, but not to the presence of a variant in *SCN5A*.

The occurrence of EADs may be linked to an abnormally high density of depolarizing late sodium current ($I_{Na,L}$) during APs repolarizing phase.⁸ Accordingly, BrS hiPSC-CMs presented with a higher density of $I_{Na,L}$ as compared to Ctrl and Non-BrS hiPSC-CMs (Figure 3C,3D). Moreover, an increase in $I_{Na,L}$ density was observed only in 6% and 12% of Ctrl and Non-BrS hiPSC-CMs respectively, in accordance with their low EAD occurrence, whereas increased $I_{Na,L}$ density was present in 50% to 85% of all BrS ventricular-like hiPSC-CMs, reminiscent of the high EAD occurrence (Figure 3B,3E). We then superfused ventricular-like BrS hiPSC-CMs during AP recording with GS-458967 (6-(4-(Trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine, which selectively blocks late sodium current),⁹ causing full inhibition of $I_{Na,L}$ (Figure 3F), and found abolishment of EADs (Figure 3G,3H) and reduced APD90 dispersion (Figure 3I). Altogether, these data strongly suggested that the abnormal increase of $I_{Na,L}$ in BrS hiPSC-CMs is responsible for EADs.

Further strengthening the role of the $I_{Na,L}$ in the electrical cellular phenotype of BrS, when each ECG parameter was tested for its correlation with either $I_{Na,L}$ or I_{Na} measured densities, only $I_{Na,L}$ density correlated significantly with one sole parameter, *i.e.*, the J point elevation (Table S5).

To challenge the pathophysiological relevance of the ion current alterations identified in Non-BrS and BrS2⁺ hiPSC-CMs, we applied them to a mathematical human electrogram model, that allows visualizing transmural-like electrogram with a QRS-like complex, a ST-like segment, and a T-like wave (Figure 4A).¹⁰ First, in accordance with BrS2⁺ patient's ECG, applying the alterations observed in peak I_{Na} , $I_{Ca,L}$ and in $I_{Na,L}$ in BrS2⁺ hiPSC-CMs was sufficient to induce prolongation of the QRS-like complex, ST-like segment elevation and widening, and T-like wave inversion (Figure 4B). Then, sequential correction of each altered current in BrS2⁺ hiPSC-CMs was made (BrS2⁺_{corrected}). Correction of I_{Na} density led to QRS-like complex normalization; correction of $I_{Ca,L}$ density shortened the duration of the ST-like segment elevation and normalized the T-like wave orientation; and correction of $I_{Na,L}$ density led to reduction of ST-like segment amplitude towards normalization (Figure 4C, left to right). Overall, these

results strongly suggest that depolarizing currents alterations can impact a multi-cellular electrogram model, mimicking BrS ECG phenotype.

In conclusion, in the present study, a particular cellular electrophysiological phenotype common to six out of six BrS hiPSC-CM lines with various genetic backgrounds has been unveiled. We showed that high EAD occurrence associates with an abnormal increase of $I_{Na,L}$ in all investigated BrS cell lines, and correlates with the corresponding patients' J point elevation on ECG. We focused on the ventricular cell type, at a single-cell level. Implementation of emerging phenotypic technologies, such as singlecell transcriptomics and cardiac tissue engineering, will allow investigation of the potential involvement of the other cardiac cell types in the disease phenotype and the role of specific cell-tocell interactions. Altogether, the obtained results open perspectives to better understand the ventricular arrhythmia occurrence in BrS and to identify a dedicated therapeutic approach to prevent the risk of SCD.

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Conflict of Interest

None.

Data availability statement.

In accordance with the "DFG Guidelines on the Handling of Research Data", the authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. The data set will be archived for at least 10 years after publication.

References

1. Priori SG, Wilde AA, Horie M, et al. HRS/EHRA/APHRS Expert Consensus Statement on the Diagnosis and Management of Patients with Inherited Primary Arrhythmia Syndromes. *Heart Rhythm*. 2013;10:1932-63.

2. Gourraud J-B, Barc J, Thollet A, et al. The Brugada Syndrome: A Rare Arrhythmia Disorder with Complex Inheritance. *Front Cardiovasc Med*. 2016;3:9.

3. Al Sayed ZR, Canac R, Cimarosti B, Bonnard C, Gourraud JB, Hamamy H, Kayserili H, Girardeau A, Jouni M, Jacob N, Gaignerie A, Chariau C, David L, Forest V, Marionneau C, Charpentier F, Loussouarn G, Lamirault G, Reversade B, Zibara K, Lemarchand P, Gaborit N. Human model of IRX5 mutations reveals key role for this transcription factor in ventricular conduction. *Cardiovasc Res.* 2020;8:259.

4. Kapplinger JD, Tester DJ, Alders M, et al. An international compendium of mutations in the SCN5A-encoded cardiac sodium channel in patients referred for Brugada syndrome genetic testing. *Heart Rhythm*. 2010;7:33-46.

5. Yan GX, Antzelevitch C. Cellular basis for the Brugada syndrome and other mechanisms of arrhythmogenesis associated with ST-segment elevation. *Circulation*. 1999;100:1660-6.

6. Meregalli PG, Wilde AA, Tan HL. Pathophysiological mechanisms of Brugada syndrome: depolarization disorder, repolarization disorder, or more? *Cardiovasc Res.* 2005;67:367-78.

7. Belbachir N, Portero V, Al Sayed ZR, Gourraud JB, Dilasser F, Jesel L, Guo H, Wu H, Gaborit N, Guilluy C, Girardeau A, Bonnaud S, Simonet F, Karakachoff M, Pattier S, Scott C, Burel S, Marionneau C, Chariau C, Gaignerie A, David L, Genin E, Deleuze JF, Dina C, Sauzeau V, Loirand G, Baró I, Schott JJ, Probst V, Wu JC, Redon R, Charpentier F, Le Scouarnec S. RRAD mutation causes electrical and cytoskeletal defects in cardiomyocytes derived from a familial case of Brugada syndrome. *Eur Heart J*. 2019 Oct 1;40(37):3081-3094.

8. Shryock JC, Song Y, Rajamani S, et al. The arrhythmogenic consequences of increasing late INa in the cardiomyocyte. *Cardiovasc Res.* 2013;99:600-11.

9. Potet F, Egecioglu DE, Burridge PW, George AL Jr. GS-967 and Eleclazine Block Sodium Channels in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *Mol Pharmacol*. 2020;98:540-547.

10. Gima K, Rudy Y. Ionic current basis of electrocardiographic waveforms: a model study. *Circ Res*. 2002;90:889-96.

Figure legends

Figure 1. Pluripotency and *SCN5A* variant validation in hiPSCs, and characterization of derived cardiomyocytes.

A. Transcript expression of pluripotency markers: NANOG and OCT3/4, in newly described hiPSCs as compared to fibroblasts (Fibro).

B. Representative immunostainings of TRA1-60 (red) and OCT4 (green) in hiPSCs.

C. Percentage of hiPSCs expressing SSEA4 and TRA1-60 evaluated by flow cytometry.

D. Genomic sequence chromatograms validating (right) the 5164A>G *SCN5A* variant carried by BrS1⁺ and (left) the *SCN5A* 1983-1993 duplication carried by BrS2⁺ and Non-BrS, in the corresponding hiPSCs. E. Principal component analysis (PCA) of 39 hiPSC samples and their corresponding differentiated hiPSC-CMs, based on their expression pattern of 27106 analyzed transcripts (3'SRP data). All clones of each hiPSC lines are highlighted.

F. Correlation matrix of hiPSCs and hiPSC-CMs expression profiles. Yellow and orange indicate high and low correlation, respectively. Samples were clustered using an ascending hierarchical method with Pearson as metric and ward.D2 linkage.

G. Heatmap showing expression levels of 9661 differentially expressed genes between hiPSCs and hiPSC-CMs (same samples as in A). Genes were clustered using a hierarchical ascending method with an uncentered correlation metric and complete linkage. Yellow and blue indicate high and low levels respectively.

H. Illustrative immunostainings of Troponin I (green) in hiPSC-CMs. Nuclei were stained with DAPI (blue).

I. Percentages of nodal-like, atrial-like, and ventricular-like cells classified based on the analysis of spontaneous action potential recordings.

Figure 2. Differential gene expression profiles and variations in I_{Na} and $I_{Ca,Lr}$ in BrS-hiPSC-CMs as compared to controls.

A. Heatmap showing hierarchical clustering of expression profiles of 133 differentially expressed genes obtained by 3'SRP in control (Ctrl) and BrS hiPSC-CMs at day 28 of differentiation. A total of 27% were upregulated whereas 73% genes were downregulated in BrS hiPSC-CMs. Yellow and blue represent high and low expression levels, respectively. All clones of each hiPSC line are highlighted.

B. Gene Set Enrichment Analysis (GSEA) of gene variations obtained by 3'SRP shows gene sets with statistically altered expression patterns.

C. MindMap describing the transmembrane transporter activity alterations.

D. Expression levels of differentially expressed genes identified using high-throughput TaqMan (TLDA) in BrS hiPSC-CMs (n=14), compared to control hiPSC-CMs (n=12), and in Non-BrS hiPSC-CMs (n=4) *vs*.

BrS hiPSC-CMs. p-values: *, ** and *** or a, b and c: p<0.05, p<0.01 and p<0.001 vs. Ctrl or BrS, respectively (*t*-test).

E. Representative immunoblots for Na_v1.5 and Transferrin receptor (TFRC) in hiPSC-CMs (left panel). Ratios of Na_v1.5 expression levels (right panel, Tukey plot, n=8). * p<0.05 *vs.* control (Mann-Whitney test). Na_v1.5 decrease in hiPSC-CMs from 3 subjects, BrS2⁺ and Non-BrS (both carrying a stop codon in *SCN5A*), as well as BrS5⁻, harboring *RRAD* variant was observed.

F. Representative superimposed I_{Na} densities (inset: voltage-clamp protocol). Reduction was detected in BrS2⁺, BrS5⁻, and Non-BrS, as well as in BrS1⁺ hiPSC-CMs carrying the N1722D-SCN5A rare variant.

G. Peak I_{Na} densities measured in control (Ctrl), BrS and the non-affected carrier of *SCN5A* mutation (Non-BrS) hiPSC-CMs determined at -20 mV (Tukey plot). *** p<0.001 *vs.* control (Mann-Whitney test). H. Mean peak I_{Na} densities (pA/pF) *vs.* membrane potential (V_m) recorded in hiPSC-CMs. ****, \$\$\$, #### and ^^^^ p<0.0001 *vs.* control for BrS1⁺, BrS2⁺, BrS5⁻ and Non-BrS, respectively (Two-way ANOVA with Bonferroni post-hoc test).

I. Representative immunoblots for Ca_v1.2, the main pore-forming subunit of the cardiac L-type calcium channel, and Transferrin receptor (TFRC) in hiPSC-CMs (left panel). Ratios of Ca_v1.2 expression levels (right panel, Tukey plot, n=8). A decrease in Ca_v1.2 expression was solely observed in BrS5⁻ hiPSC-CMs, carrying a *RRAD*-variant. * p<0.05 *vs.* control (Mann-Whitney test).

J. Representative superimposed I_{Ca,L} densities (inset: voltage protocol).

K. Peak $I_{Ca,L}$ densities measured in control (Ctrl), BrS and the non-affected carrier of *SCN5A* mutation (Non-BrS) hiPSC-CMs determined at 0 mV (Tukey plot). A decrease in $I_{Ca,L}$ was observed in BrS2⁺, BrS4⁻ and, consistently with a previous description, in BrS5⁻.⁷ * p<0.05 and ** p<0.01 *vs.* control (Mann-Whitney test).

L. Mean peak $I_{Ca,L}$ densities (pA/pF) vs. membrane potential (V_m) recorded in hiPSC-CMs. *, # and \$ p<0.05, **, ## and \$\$ p<0.01 and ***, ### and \$\$\$ p<0.001 vs. control for BrS1⁺, BrS4⁻ and BrS5⁻, respectively (Two-way ANOVA with Bonferroni post-hoc test).

Figure 3. Increased early after depolarization (EAD) occurrence in all BrS ventricular-like hiPSC-CMs lines, linked to an increase in late sodium current.

A. Representative AP recordings, showing EADs in BrS lines only. Representative ventricular-like AP when paced at 700 ms cycle length and when artificial I_{K1} was injected (dynamic current-clamp). APs are defined as ventricular-like when $(APD_{30}-APD_{40})/(APD_{70}-APD_{80})>1.45$.

B. Percentage of ventricular-like hiPSC-CMs presenting at least 1 EAD, irrespective of the currentclamp conditions. * p<0.05, ** p<0.01 and *** p<0.001 vs. control (Fisher's exact test).

C. Representative $I_{Na,L}$ recordings from hiPSC-CMs, before (black) and after (grey) TTX application (inset: voltage protocol).

D. $I_{Na,L}$ (TTX-sensitive current) densities at -10 mV. * p<0.05 and *** p<0.001 vs. Ctrl (Mann-Whitney test), and ^{##} p<0.01 and ^{###} p<0.001 vs. Non-BrS (Mann-Whitney test).

E. Percentage of cells presenting $I_{Na,L}$ density greater than the 97th percentile value of $I_{Na,L}$ in the Ctrl hiPSC-CMs. ** p<0.01 and *** p<0.001 *vs*. control (Fisher's exact test). Indeed, an increase in $I_{Na,L}$ density was defined by values higher than the 97th percentile of the Ctrl hiPSC-CMs.

F. Representative example of $I_{Na,L}$ current recorded in BrS hiPSC-CMs before (black) and after (red) application with GS-458967 (300 nM), a specific $I_{Na,L}$ inhibitor (inset: voltage protocol).

G. Representative AP recordings from control and BrS hiPSC-CMs obtained before and after GS-458967 application.

H. Percentage of cells with EADs before and after GS-458967 application.

 Poincaré plots showing APD₉₀ of each AP (n+1) vs. APD₉₀ of its preceding one, before and after GS-458967 application.

Figure 4. Applying depolarizing ion current alterations from BrS hiPSC-CMs on an electrogram model to mimic BrS patient's ECG features.

A. Top: The right-ventricle electrogram model simulates the global electrical activity of a transmural wedge comprising 60 subendocardial, 45 midmyocardial, and 60 subepicardial human ventricular cells. Bottom: Representative electrogram showing the Q-like, R-like, S-like and T-like waves.

B. Ventricular transmural electrogram mathematical model of Ctrl (black), BrS2⁺ (pink) and Non-BrS (blue) illustrated based on hiPSC-CMs data of the relative variation in ion currents (I_{Na} , $I_{Ca,L}$ and $I_{Na,L}$) mean amplitude as compared to Ctrl lines. In accordance with patient's ECGs, applying BrS2⁺ ionic current changes prolonged the QRS-like complex, elevated and widened the ST-like segment, and inversed the T-like wave; and applying I_{Na} change identified in Non-BrS hiPSC-CMs only prolonged the QRS-like complex, similar to Non-BrS PCCD ECG.

C. Each currents I_{Na} , $I_{Ca,L}$ and $I_{Na,L}$ (from left to right) were sequentially corrected and the resulting electrograms are illustrated in green. Ctrl and BrS2⁺ electrograms are presented in black and pink respectively.



Figure 1. Pluripotency and SCN5A variant validation in hiPSCs, and characterization of derived cardiomyocytes.

179x164mm (600 x 600 DPI)



Figure 2. Differential gene expression profiles and variations in INa and ICa,L, in BrS-hiPSC-CMs as compared to controls.

229x189mm (600 x 600 DPI)



Figure 3. Increased early after depolarization (EAD) occurrence in all BrS ventricular-like hiPSC-CMs lines, linked to an increase in late sodium current.

179x119mm (600 x 600 DPI)



Figure 4. Applying depolarizing ion current alterations from BrS hiPSC-CMs on an electrogram model to mimic BrS patient's ECG features.

129x120mm (600 x 600 DPI)

A consistent arrhythmogenic trait in Brugada syndrome cellular phenotype

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SUPPLEMENTAL MATERIAL

Supplemental methods:

Ethical Statement

The study was conducted according to the principles set forth under the Declaration of Helsinki (1989) and European guidelines for clinical and genetic research. Institutional review board approvals of the study were obtained before the initiation of patient enrollment. The study protocol was reviewed and approved by the regional *"comité de protection des personnes"* ethical committee (approval number: 2010-A01358-31). Regarding the patient-derived biological samples, signed informed consent allowing the experiments to be conducted has been received from all individuals. Any related health information was collected in compliance with applicable law/regulation and with any applicable policy of the ethics committee with jurisdiction over the biological sample collection. All biological samples and their related health information have been provided in coded form such that subjects cannot be identified directly. The provisions of French law, article L1110-4 of the *Code de la santé publique*, related to the privacy and confidentiality of information regarding patients, have been observed.

Study Population and design

BrS patients were enrolled according to the presence of a BrS ECG pattern (see below) and with a familial history of sudden death or syncope. Diagnosis of BrS was based on criteria from 2013¹ with the presence of a type-1 BrS ECG pattern, either spontaneous or induced by intravenous injection of class I antiarrhythmic drugs, in at least one right precordial lead (V1 or V2) positioned in the 2nd, 3rd or 4th intercostal space. Type-1 BrS ECG pattern was defined as a J point elevation higher than 0.2 mV, followed by a coved type ST segment elevation and ended with a negative T wave.

ECG analysis

Two physicians blinded to the clinical and genetic status reviewed all baseline ECGs. P wave, PQ interval, QRS, QT peak, QTend, QTc duration (corrected by Bazett formula), and Tpeak-Tend interval (TPE, time interval between the peak and the end of the T wave) were measured in D2, V1, V2 and V3. S-wave duration and amplitude were additionally measured in D1. All measurements were performed using Image J software as previously described.^{2,3} TPE in precordial lead, fragmented QRS, early-repolarization pattern and prominent R wave in lead aVR were determined as previously described.^{4,5} *Genetic analysis*

Patients were screened for mutations in genes previously described in cardiomyopathies and inherited arrhythmias.^{6,7} For this, genomic DNA of probands was extracted from peripheral blood lymphocytes by standard protocols. The DNA yields were assessed by measurements using Quant-IT[™] dsDNA Assay Kit, Broad Range (Life Technologies, Q33130). The purity of the DNA was assessed by

spectrophotometry (OD 260:280 and 260:230 ratios) using a Nanodrop instrument (Thermo Scientific). DNA integrity was assessed by separation in E-Gel[®] 96 Agarose Gels, 1% (Life Technologies, G700801). For multiplex amplification, we used the HaloPlex[™] Target Enrichment System (Agilent Technologies, 1-500 kb, ILMFST, 96 reactions, G9901B, Protocol Version D.2 (November, 2012)). A custom HaloPlex[™] design was used enabling high-throughput sequencing of the coding regions (exons ± 10 bp) of 20 genes previously associated with the BrS:^{6,7}

SCN5A	CACNA1C	RANGRF	KCNE1L
GPD1L	CACNB2	РКР2	KCNJ8
SCN1B	CACNA2D1	FGF12	ABCC9
SCN2B	KCNE3	SCN10A	KCNH2
SCN3B	KCND3	TRPM4	HCN4

Target enrichment and sequencing were performed as previously described.⁷ First, 200 ng of gDNA sample were digested in eight different restriction reactions, each containing two restriction enzymes, to create a library of gDNA restriction fragments. These gDNA restriction fragments were hybridized to the HaloPlex probe capture library. Probes were designed to hybridize and circularize targeted DNA fragments. During the hybridization process, Illumina sequencing motifs including index sequences were incorporated into the targeted fragments.

The circularized target DNA biotinylated HaloPlex probe complexes were captured on magnetic streptavidin beads. We proceeded to a ligation reaction of the circularized complexes followed by an elution reaction before PCR amplification. The amplified target DNA was purified using AMPure XP bead (Beckman Coulter, A63881). To validate enrichment of target DNA in each library sample by microfluidics analysis, we used the 2200 TapeStation (Agilent Technologies, G2964AA), with D1K ScreenTape (Agilent Technologies, 5067-5361), and D1K Reagents (Agilent Technologies, 5067-5362). We ensured that the majority of amplicons range from 175 to 625 bp. Finally, we quantified the library together with other libraries by qPCR using the KAPA Library Quantification Kit (Clinisciences, KK4854). Libraries were pooled in an equimolar concentration and DNA was then denatured with NaOH. Finally, the library pool was diluted to a final concentration of 9 pM before proceeding to 100-bp paired-end Illumina sequencing on HiSeq1500.

Raw sequence reads were aligned to the human reference genome (GRCh37) using BWA-MEM (version 0.7.5a) after removing sequences corresponding to Illumina adapters with Cutadapt v1.2. GATK was used for insertions and deletions (indel) realignment and base recalibration, following GATK DNAseq Best Practices. Variants were called for each sample separately using the Genome Analysis Toolkit GATK (UnifiedGenotyper version 2.8) Variants were considered as rare if the frequency was < 0.01% compared with the Gnomad database.⁸

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Variants were considered as having a potential functional consequence if they were annotated with one or more of the following SO terms for at least one RefSeq transcript: "transcript ablation" (SO:0001893), "splice_donor_variant" (SO:0001575), "splice_acceptor_variant" (SO:0001574), "stop_gained" (SO:0001587), "frameshift_variant" (SO:0001589), "stop_lost" (SO:0001578), "initiator_codon_variant" (SO:0001582), "inframe_insertion" (SO:0001821), "inframe_deletion" (SO:0001822), "missense_variant" (SO:0001583), "transcript_amplification" (SO:0001889). Loss-offunction variants (nonsense variants, frameshift variants and splice site variants) were defined by the following SO terms: "stop gained", "frameshift variant", "splice donor variant", or "splice_acceptor_variant". The potential pathogenicity of variants was determined following the American College of Medical Genetics and Genomics (ACMG) guidelines using the CardioVAI tools from EnGenome.9

Generation and validation of hiPSCs

BrS5⁻ hiPSCs were previously used in a study revealing the role of a variant in *RRAD*, which encodes RAD GTPase, in BrS.¹⁰ BrS6⁻ hiPSC line, named iBrS1 in a previous study, was derived from a BrS patient with no defined causative variant.⁶ Four different control hiPSC lines were used, including 2 studied previously.^{10,11} BrS4⁻ cutaneous fibroblasts were reprogrammed using Stemgent mRNA reprogramming kit and pluriton medium. hiPSCs derived from dermal cells harvested from BrS1⁺ and BrS3⁻ as well as blood cells obtained from BrS2⁺ and his unaffected family relative (Non-BrS) were reprogrammed using Sendai virus method (Cytotune reprogramming Kit, Life technologies). Up to three clones of each hiPSC line were selected per patient. HiPSCs were maintained on matrigel-coated plates (0.05 mg/ml, BD Biosciences) with StemMACSTM iPS-Brew XF medium (Miltenyi Biotec). Pluripotency of each hiPSC clone was validated by qRT-PCR, immunostaining, and flow cytometry to verify the expression of endogenous pluripotent factors.

Validation of SCN5A rare variants and genome integrity

To validate *SCN5A* rare variants carried by BrS1⁺, BrS2⁺ and Non-BrS, genomic DNA from corresponding hiPSC clones was extracted using NucleoSpin® Tissue kit (MACHEREYNAGEL). Variants were verified by sequencing. Single nucleotide polymorphism (SNP) analysis of all hiPSC clones compared to their parental skin fibroblast cells, was used to confirm genome integrity after reprogramming. DNA was extracted from somatic and hiPSC samples using the QIAGEN QiaAmp kit, according to the manufacturer's recommendations. The gDNA was quantified using a Nanodrop instrument. 200ng of gDNA were outsourced to Integragen Company (Evry, France) for karyotype analysis using HumanCore-24-v1 SNP arrays. This array contains over 300,000 probes distributed throughout the genome with a median coverage of one probe every 5700 bases. All genomic positions were based on Human Genome Build 37 (hg19). Analysis was performed with GenomeStudio software. Chromosome abnormalities were determined by visual inspection of logR ratios and B-allele frequencies (BAF) values and

comparing parental cells with hiPSC-derived samples. LogR ratio, the ratio between observed and expected probe intensity, is informative of copy number variation (CNV, i.e. deletions/duplications), whereas BAF is informative of heterozygosity. SNP data were used to compute CNV. In particular, this type of chips allows detecting loss of heterozygosity (LOH), an important concern for hiPSCs, which is not detectable with classical CGH arrays.

Differentiation of hiPSCs into cardiomyocytes

Cardiomyocytes were differentiated from hiPSCs using the established matrix sandwich method.¹¹ Briefly, when cells reached 90% confluence, an overlay of Growth Factor Reduced Matrigel (0.033 mg/ml, BD Corning) was added. Differentiation was initiated 24 h later by culturing the cells in RPMI1640 medium (Life Technologies) supplemented with B27 (without insulin, Life Technologies), 2 mM L-glutamine (Life Technologies), 1% NEAA (Life Technologies), 100 ng/mL Activin A (Miltenyi), and 10 ng/mL FGF2 for 24 hours. On the next day, the medium was replaced by RPMI1640 medium supplemented with B27 without insulin, 2 mM L-glutamine, 1% NEAA, 10 ng/mL BMP4 (Miltenyi), and 5 ng/mL FGF2 for 4 days. By day 5, cells were cultured in RPMI1640 medium supplemented with B27 complete (Life Technologies), 2 mM L-glutamine and 1% NEAA and changed every two days. Presence of beating cells was considered as the primary hallmark of a successful cardiomyocyte differentiation.

Transcript expression analysis

Quantitative RT-PCR: Total RNA samples were isolated using the NucleoSpin RNA kit (MACHEREY-NAGEL). One μ g of tRNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. PCR amplification was performed using FAM labeled-TaqMan probes (Applied Biosystems) to verify the pluripotency of derived hiPSCs as compared to parental fibroblasts (OCT3/4 and NANOG). According to the $\Delta\Delta$ Ct method, all data were normalized to ACTB and represented relative to controls.

TaqMan Low Density array (TLDA): TLDA studies were conducted using beating clusters of hiPSC-CMs obtained from 12 samples of Ctrl, 4 samples of Non-BrS and 14 samples of BrS hiPSC-CMs. One μ g of RNA was reverse transcribed into cDNA using SuperScript IV Vilo Master Mix (Thermo Fisher Scientific). TLDA probe selection covered gene families implicated in cardiac ion channel expression and regulation, and cardiomyocyte structure (Table S3). Genes with average Ct > 32 in all compared groups were considered undetectable and excluded from the analysis (SCN10A and ABCC8). Average Ct of remaining genes for each sample was used for data normalization.¹²⁻¹⁴

3' Sequencing RNA Profiling (3'SRP): 3'SRP protocol was performed according to Kilens et al.¹⁵ Briefly, the libraries were prepared from 10 ng of total RNA. RNA samples were extracted from 26 BrS and 13 control hiPSC samples (a duplicate for each clone obtained at different cell passages) as well as their corresponding differentiated hiPSC-CMs. The mRNA poly(A) tail was tagged with universal adapters, well-specific barcodes and unique molecular identifiers (UMIs) during template-switching reverse

transcriptase. Barcoded cDNAs from multiple samples were then pooled, amplified and tagmented using a transposon-fragmentation approach which enriches for 3'ends of cDNA. A library of 350-800 bp was run on an Illumina HiSeq 2500 using a HiSeq Rapid SBS Kit v2 (50 cycles; FC-402-4022) and a HiSeq Rapid PE Cluster Kit v2 (PE-402-4002). Read pairs used for analysis matched the following criteria: all sixteen bases of the first read had quality scores of at least 10 and the first six bases correspond exactly to a designed well-specific barcode. The second reads were aligned to RefSeq human mRNA sequences (hg19) using bwa version 0.7.17. Reads mapping to several transcripts of different genes or containing more than 3 mismatches with the reference sequences were filtered out from the analysis. Digital gene expression profiles were generated by counting the number of UMIs associated with each RefSeq genes, for each sample. R package DESeq2 (Bioconductor) was used to normalize gene expression, and detect differentially expressed genes. Sample correlation matrix was performed using R package ComplexHeatmap (Bioconductor). Cluster 3.0 software was used to perform the differentially expressed gene heatmap. Gene Set Enrichment Analysis (GSEA) was performed using GeneTrail2. Statistically significant categories within the GO Molecular Process were identified using Kolomogorov-Smirnov test and p values were adjusted using the Benjamini and Hochberg method.

Protein expression analysis

Beating clusters of hiPSC-CMs were lysed in buffer composed of 1% TritonX-100, 100 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 1 mM PMSF and protease inhibitors cocktail (P8340, Sigma-Aldrish). Protein quantification was then conducted using PierceTM BCA Protein Assay Kit (Thermo Fisher). The lysates were denatured for 5 min at 65°C in a mixture of NuPAGE® Sample Reducing Agent (10X) and NuPAGE® LDS Sample Buffer (4X). 40 µg of each sample were loaded onto 12% precast polyacrylamide gels (Bio-Rad). After migration, the proteins were transferred onto Trans-Blot® TurboTM Nitrocellulose Transfer Packs (Bio-Rad). Membranes were saturated with 5% non-fat milk, then incubated with primary antibody (Anti-Na_v1.5: 14421S Cell Signaling; Anti-Cav1.2: AB5156 Sigma-Aldrich; Anti-TFRC:13-6890 Thermofisher) overnight followed by another incubation for 1 h with an adequate Horseradish peroxidase (HRP)-conjugated secondary antibody. Protein bands were detected using ECL detection system (Bio-Rad) and quantified using Image Lab software.

Immunostainings

To validate hiPSC pluripotency, hiPSC-CM differentiation into cardiac lineage as well as COS-7 cell expression, cells were dissociated and seeded onto 8-wells ibidi plates (Biovalley) coated with Matrigel (Corning). After 12 days following hiPSC-CM dissociation, and 24 h following COS-7 transfection, cultured cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X100 and blocked with 1% BSA. Immunofluorescent stainings were performed using appropriate primary antibodies (Anti-TRA1-60: 14-8863-80 eBioscience[™]; Anti-OCT4: 14-5841-80 eBioscience[™]; Anti-troponin I: sc-

15368 Santa Cruz; Anti-Na_v1.5: 14421S Cell Signaling) and their corresponding Alexa conjugated antibodies (Molecular Probes). DAPI was used for nuclear staining. Immunostainings were examined using an inverted epifluorescent microscope (Zeiss Axiovert 200M).

p.N1722D Nav1.5 study in COS-7 cells

The point mutation p.N1722D (c.1859G>A) in *SCN5A* was introduced using mutated oligonucleotide extension (QuikChange II XL Site-Directed Mutagenesis Kit) in *SCN5A* isoform 2 cDNA (GenBank Acc. Nb. NM_000335). The resulting plasmid was verified by complete sequencing of the cDNA insert. African green monkey kidney-derived cells COS-7, were transiently transfected with 0.4 μ g *SCN5A*-expressing plasmid (wild type or mutant) together with 0.4 μ g of Nav β 1 subunit (SCN1B) plasmid for a 35-mm Petri dish. Transfections were performed using 4 μ L JetPEI reagent (Polyplus Transfections, France) according to the manufacturer's instructions. Enhanced green fluorescent protein (eGFP)-encoding plasmid (1.2 μ g) was included to identify transfected cells. One day after transfection, cells were re-plated onto 35-mm Petri dishes for patch clamp experiments.

Electrophysiological assessment

Sodium current (I_{Na}) recordings in transfected COS-7 cells. Currents were recorded 2 days after transfection. Cells were superfused with a solution containing the following (in mM): 145 NaCl, 4 CsCl, 1 CaCl2, 1 MgCl2, 5 HEPES, and 5 glucose, pH=7.4 with NaOH. Patch pipettes were fabricated from borosilicate glass capillaries and had resistances between 1.5 and 2 MΩ when filled with pipette solution (in mM): 90 KCl, 45 K-Gluconate, 10 NaCl and 10 HEPES, pH=7.2 with CsOH. All recordings were made at room temperature (20°C-22°C), after capacitance and series resistance compensation, using an Axopatch 200B amplifier controlled by Axon pClamp 10.6 software through an A/D converter (Digidata 1440A). Data were analyzed using Clampfit 10.6 software (all Molecular Devices).

Current- and voltage-clamp in hiPSC-CMs. Cardiomyocytes were dispersed as single cells around day 20 of differentiation, for 20 min in collagenase II (200 U/mL; Gibco) at 37°C. Ten to twelve days after dissociation, spontaneously beating cells were used for patch-clamp recordings. All experiments were conducted at 37°C. Data were collected from a minimum of 3 independent differentiations.

Action potential (AP) recordings. Using amphotericin-B perforated-patch configuration, APs were acquired with the same amplifier and converter as above in hiPSC-CMs cells bathed in a Tyrode solution containing (in mM): 140 NaCl, 4 KCl, 1 CaCl2, 0.5 MgCl2, 10 glucose, 10 HEPES; pH 7.4 (NaOH). Borosilicate glass pipettes (2-3 M Ω of tip resistance) were filled with a solution containing (in mM): 125 K-Gluconate, 20 KCl, 5 NaCl, 5 HEPES; pH 7.2 (KOH) and 0.22 amphotericin-B. We first recorded spontaneous APs in order to determine, for each individual included in the study, the proportion of nodal-like, atrial-like and ventricular-like, as previously described.¹¹ Then to overcome limited I_{K1} contribution during hiPSC-CM AP, artificial I_{K1} was injected using dynamic patch-clamp.¹⁶ Both cell stimulation and I_{K1} injection were realized using a custom-made software running on RT-Linux and an

A/D converter (National Instrument PCI-6221) connected to the current command of the amplifier. The AP parameters measured were the maximum diastolic potential (MDP), the maximum upstroke velocity of phase 0 depolarization (dV/dt_{max}), the AP amplitude and the AP duration at different levels of full repolarization, from 30% (APD30) to 90% (APD90), as previously described.¹¹ Following I_{K1} injection, at a peak outward density that sets the membrane potential between -80 to -85mV for all cells, action potential classification into ventricular type was assessed by (APD30-APD40)/(APD70-APD80)>1.45 reflecting the presence of a plateau phase. Cells were paced with a 1-ms 30-35 pA/pF stimulation pulse at 700 ms of cycle length. BrS being a ventricular arrhythmic disease, the analyses were focused on ventricular-like AP. Data from 7 consecutive APs were averaged. GS-458967 (Gilead Sciences) specifically inhibiting I_{Na,L}, was solubilized in DMSO and used at 300 nM during AP recording in a solution containing (in mM): 140 NaCl, 4 KCl, 1 CaCl2, 0.5 MgCl2, 30 mannitol, 10 HEPES; pH 7.4 (NaOH).

Current recordings. I_{Na} and $I_{Ca,L}$ measurements were recorded in the ruptured-patch configuration and low-pass filtered at, respectively, 10 KHz and 3 KHz using a VE-2 amplifier (Alembic Instrument, Qc, Canada). Cells were bathed using a Tyrode solution containing (in mM): 130 NaCl, 10 CsCl, 1.8 CaCl2, 1.2 MgCl2, 11 glucose and 5 HEPES; pH 7.4 (NaOH). Holding potentials were set to -80 mV and -100 mV, respectively. The series resistance was compensated. Current densities and gating properties were measured using appropriate voltage protocols shown in the relevant figures. After leak subtraction, current densities were calculated by dividing current amplitude by membrane capacitance. Voltage-dependence of activation and inactivation curves were fitted with a Boltzmann function (y=[1+exp{-(V-V1/2)/K}]-1), where V1/2 is the half-maximal voltage of (in)activation and K is the slope factor.

For transient **sodium current (I_{Na})** recording, a local gravity microperfusion system allowed application of an extracellular solution containing (in mM): 20 NaCl, 110 CsCl, 1.8 CoCl2, 1.2 MgCl2, 30 mannitol and 5.0 HEPES; pH 7.4 (CsOH). The pipette solution contained (in mM): 3 NaCl, 133 CsCl, 2 MgCl2, 2 Na2ATP, 2 TEACl, 10 EGTA, 5 HEPES; pH 7.2 (CsOH).

Late sodium current $(I_{Na,L})$ was measured as a TTX-sensitive current (Tetrodotoxin Citrate, TOCRIS Bioscience) using an ascending voltage-ramp protocol. The same pipette solution as for peak I_{Na} recording was used. The extracellular solution used in the local gravity microperfusion system had the following composition (in mM): 130 NaCl, 10 CsCl, 1.8 CoCl2, 1 MgCl2, 30 mannitol, 10 HEPES; pH 7.4 (CsOH). TTX was used at a concentration of 0.03 mM.

Calcium current (I_{Ca,L}) was recorded from cells perfused an extracellular solution containing (in mM): 160 TEACI, 5 CaCl2, 1 MgCl2, 1 MgCl2, 20 mannitol, 10 HEPES, and 0.01 TTX; pH 7.4 (CsOH). The pipette solution contained (in mM): 5 NaCl, 145 CsCl, 2 CaCl2, 5 EGTA, 5 MgATP, 10 HEPES; pH 7.2 (CsOH).

Mathematical electrogram modeling

Right-ventricle electrogram was calculated as in Gima and Rudy,¹⁷ by simulating a heterogeneous transmural wedge (right ventricular outflow tract, 1 Hz, 500th beat shown). This model aims at mimicking the global electrical activity of a row of 165 subendocardial, midmyocardial and subepicardial human ventricular cells.^{18,19} In the original model used by Gima and Rudy, a slow inactivation gate (j) of the Na+ channel was added to include the property of slow recovery,²⁰ without modifying fast inactivation (realized with h gate). A few modifications were operated. Since this 'j' inactivation was originally as fast as 'h' inactivation, it prevents the appearance of a persistent current when altering the 'h' gate. So we slowed down this 'j' inactivation, but left the kinetics of recovery intact, by modifying the following equation:

If $V \ge -50$ beta_j=0.035*exp(+0.008*p->v)/(1.0+exp(-0.048*(p->v+77.329)).

Thus, in the modified model, any incomplete fast inactivation (for instance, h parameter varies between 1 and 0.166 for BrS2⁺, instead of 1 and zero for Ctrl) gives rise to a late Na⁺ current similar to that observed in Figure 3C (see also Figure below) resulting in the following equation.



Impact of incomplete inactivation on late sodium current in a simulated Ramp protocol. Ctrl: h parameter varies between 1 and 0.1^7 BrS: h varies between 1 and 0.116. The ratio of maximal I_{Na,L} current in BrS/Ctrl is 5.4, as in Figure 3C for BrS2⁺.

Noteworthy, this modification does not change inactivation properties of the Na⁺ current in the WT model because fast inactivation (h) is not modified.

Since only a subset of hiPSC-CMs present the late Na⁺ current, we performed simulations with the late Na⁺ current only in midmyocardial cells, giving rise to a J point elevation followed by coved ST segment.¹⁸

Since decreasing I_{Na} by as much as 73-75% (BrS2⁺ and Non-BrS) was preventing conduction in the Gima and Rudy model, a slighter 30% decrease in I_{Na} was applied.

Statistical analysis

Results are expressed as mean \pm SEM. Comparisons were made by use of Mann-Whitney test, Student *t*-test, or two-way ANOVA with Bonferroni post-hoc test for repeated measures. Correlations were investigated based on correlation coefficient r_s of Spearman.

Values of p < 0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism software.

Supplemental results:

Patient characteristics

Six patients affected by type I BrS (BrS1-6) with a familial history of SCD or syncope were selected, among which two carrying *SCN5A* variants (marked with a ⁺ symbol in BrS1⁺ and BrS2⁺).

Patient #1 (BrS1⁺) was a 35-year-old male with a history of recurrent near-syncope. He presented with a spontaneous BrS ECG pattern. After induction of ventricular fibrillations (VF) during electrophysiological study, an implantable cardioverter-defibrillator (ICD) was implanted with no recurrence of syncope or VF during a 14-year follow-up. Five of his relatives also exhibited a BrS ECG pattern.

Patient #2 (BrS2⁺) was an asymptomatic 55-year-old male whose brother died suddenly at age 38. He presented a spontaneous BrS ECG pattern with occurrence of VF during an electrophysiological study. An ICD was implanted with no occurrence of syncope or VF during a 13-year follow-up. Six of his relatives presented with a BrS ECG pattern including his son who exhibited one spontaneous episode of VF.

Patient #3 (BrS3⁻) was a 35-year-old male who suffered from syncope at night and presented with a spontaneous BrS ECG pattern with VF occurrence during an electrophysiological study. Despite hydroquinidine administration, several episodes of VF were reported. Endocardial catheter ablation of premature ventricular beats was performed without further recurrences under hydroquinidine therapy. Two of his relatives presented with a BrS ECG pattern.

Patient #4 (BrS4⁻) was a 44-year-old male who presented episodes of unexplained syncope at rest. Ajmaline test revealed a BrS ECG pattern. Electrophysiological study did not induce any arrhythmia. Due to familial history of SCD and recurrent syncope, an ICD was implanted without recurrence of syncope or VF during a 13-year follow-up. Type-1 BrS ECG was identified in 8 additional family members.

Patient #5 (BrS5⁻) was a 41-year-old male, previously described by Belbachir *et al*,¹⁰ who presented recurrent near-syncopes with palpitations and spontaneous BrS ECG pattern. Electrophysiological study induced VF. An ICD was implanted with no recurrence of syncope or VF during a 16-year follow-up. Familial screening identified 6 relatives with a BrS ECG pattern and one with unexplained SCD at age 41.

Patient #6 (BrS6⁻) was a 42-year-old male, previously described by Veerman *et al.*⁶ While he had a spontaneous BrS ECG pattern, he presented an unexplained syncope, and suffered from an out-of-hospital cardiac arrest at night. Three of his relatives presented with a BrS phenotype after ajmaline administration.

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An additional Patient #7, not affected by Brugada syndrome (Non-BrS), was recruited. He was a 67-year-old male diagnosed with progressive cardiac conduction defect (PCCD). He was the nephew of patient BrS2⁺. His ECG displayed broad PR interval and prolonged QRS duration, however, no ST segment elevation was detected even after challenge with flecainide.

Four control subjects were also included. One subject who was a relative of BrS5⁻ patient,¹⁰ in whom BrS was excluded after sodium channel blocker challenge and three other unrelated healthy extra familial controls, including one previously described¹¹ and 2 others with different ethnicity.²¹

Representative patient ECGs are presented in Figure S1. Description of patients and their corresponding ECG measurements are depicted in Table S1 and Table S2 respectively.

Genetic characterization

Genetic screening of the coding regions of 20 genes, selected according to the American College of Medical Genetics and Genomics (ACMG) guidelines, revealed *SCN5A* pathogenic variants in only two of the six BrS patients. BrS1⁺ carried a c.5164A>G missense rare variant, resulting in asparagine to aspartic acid substitution at position 1722 in the extracellular connecting loop, between segment 5 and 6 in domain IV forming the pore region of Na_v1.5 (p.N1722D). BrS2⁺ and his nephew (Non-BrS) affected by PCCD carried a 10bp duplication (c.1983–1993dup) in *SCN5A* creating a stop codon (p.A665G-fsX16). Both these variants had not been functionally investigated yet. BrS5⁻ patient presented a previously described rare genetic variant in the *RRAD* gene (p.R211H)¹⁰ whereas no genetic variation was identified in BrS candidate genes in BrS3⁻, BrS4⁻ or BrS6⁻ patients (Table S1).

Generation and characterization of hiPSCs and hiPSC-CMs

Somatic cells were obtained from all studied subjects and were reprogrammed into corresponding hiPSC lines. BrS5⁻, BrS6⁻ and the control hiPSC lines have been previously characterized.^{6,10,11,21} For each of the other 5 newly generated hiPSC lines (BrS1 to 4 and Non-BrS), up to three independent clones were amplified and characterized. SNP analysis verified that control and mutated hiPSC lines were free from any genomic aberrations compared to parental somatic cells (data not shown). The expression of the pluripotent stem cell markers was verified (Figure 1A, 1B and 1C). Genetic screening confirmed that the lines arising from patients carrying *SCN5A* genetic variants, BrS1⁺, BrS2⁺ and Non-BrS harbored the corresponding heterozygous rare variant (Figure 1D).

hiPSC differentiation process into cardiomyocytes was also validated at the transcriptional level. 3'SRPbased global transcriptomic analysis of control and BrS hiPSCs and hiPSC-CMs showed that cells clustered based on their stage (hiPSCs on one side and hiPSC-CMs on the other side) and independently from their genetic background (Figure 1E). Correlation analysis also showed that samples correlated according to their stage, with all hiPSC samples being correlated to other hiPSC samples and similarly for hiPSC-CM samples (Figure 1F). Finally, both Ctrl and BrS hiPSC-CMs presented a comparable global change in gene expression as compared to hiPSC stage (Figure 1G). Immunostaining analysis showed

that striated troponin I, a cardiac and muscular specific cytoskeletal protein, was similarly present in all hiPSC-CM lines (Figure 1H). Spontaneous APs, in absence of I_{K1} injection, were classified based on MDP, dV/dt_{max}, AP duration and morphology into nodal-like, atrial-like and ventricular-like, as previously described.¹¹ The proportion of each cell type was similar between all hiPSC-CM lines, with the ventricular-like type forming the majority of explored hiPSC-CMs (Figure 1I). Altogether, these data confirmed that Ctrl, BrS, and Non-BrS hiPSCs differentiated similarly into cardiomyocytes, and therefore a comparative electrophysiological analysis could be performed to unveil a potential common cellular phenotypic trait of BrS hiPSC-CMs.

Supplemental references:

1. Priori SG, Wilde AA, Horie M, et al. HRS/EHRA/APHRS Expert Consensus Statement on the Diagnosis and Management of Patients with Inherited Primary Arrhythmia Syndromes. *Heart Rhythm*. 2013;10:1932-63.

2. Therasse D, Sacher F, Babuty D, et al. Value of the sodium-channel blocker challenge in Brugada syndrome. *Int J Cardiol*. 2017;245:178-80.

3. Therasse D, Probst V, Gourraud J-B. Sodium channel blocker challenge in Brugada syndrome: Role in risk stratification. *Int J Cardiol*. 2018;264:100-1.

4. Gourraud J-B, Barc J, Thollet A, et al. Brugada syndrome: Diagnosis, risk tratification and management. *Arch Cardiovasc Dis.* 2017;110:188-95.

5. Berthome P, Tixier R, Briand J, et al. Clinical presentation and follow-up of women affected by Brugada syndrome. *Heart Rhythm.* 2018;16:260-267.

6. Veerman CC, Mengarelli I, Guan K, Stauske M, Barc J, Tan HL, Wilde AAM, Verkerk AO, Bezzina CR. hiPSC-derived cardiomyocytes from Brugada Syndrome patients without identified mutations do not exhibit clear cellular electrophysiological abnormalities. *Sci Rep.* 2016;6:30967.

7. Scouarnec SL, Karakachoff M, Gourraud J-B, Lindenbaum P, Bonnaud 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, Haïssaguerre M, Kyndt F, Schmitt S, Bézieau S, Marec HL, Dina C, Schott J-J, 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;ddv036.

8. Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, Collins RL, Laricchia KM, Ganna A, Birnbaum DP, Gauthier LD, Brand H, Solomonson M, Watts NA, Rhodes D, Singer-Berk M, Seaby EG, Kosmicki JA, Walters RK, Tashman K, Farjoun Y, Banks E, Poterba T, Wang A, Seed C, Whiffin N, Chong JX, Samocha KE, Pierce-Hoffman E, Zappala Z, O'Donnell-Luria AH, Minikel EV, Weisburd B, Lek M, Ware JS, Vittal C, Armean IM, Bergelson L, Cibulskis K, Connolly KM, Covarrubias M, Donnelly S, Ferriera S, Gabriel S, Gentry J, Gupta N, Jeandet T, Kaplan D, Llanwarne C, Munshi R, Novod S, Petrillo N, Roazen D, Ruano-Rubio V, Saltzman A, Schleicher M, Soto J, Tibbetts K, Tolonen C, Wade G, Talkowski ME, Consortium TGAD, Neale BM, Daly MJ, MacArthur DG. Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. *bioRxiv*. 2019;531210.

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, 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 Off J Am Coll Med Genet*. 2015;17:405-24.

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10. Belbachir N, Portero V, Al Sayed ZR, et al. RRAD mutation causes electrical and cytoskeletal defects in cardiomyocytes derived from a familial case of Brugada syndrome. *Eur Heart J.* 2019;ehz308.

11. Es-Salah-Lamoureux Z, Jouni M, Malak OA, et al. HIV-Tat induces a decrease in IKr and IKs via reduction in phosphatidylinositol-(4,5)-bisphosphate availability. *J Mol Cell Cardiol*. 2016;99:1-13.

12. Wang W-X, Danaher RJ, Miller CS, Berger JR, Nubia VG, Wilfred BS, Neltner JH, Norris CM, Nelson PT. Expression of miR-15/107 family microRNAs in human tissues and cultured rat brain cells. *Genomics Proteomics Bioinformatics*. 2014;12:19-30.

13. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol.* 2009;10:R64.

14. Bockmeyer CL, Säuberlich K, Wittig J, Eßer M, Roeder SS, Vester U, Hoyer PF, Agustian PA, Zeuschner P, Amann K, Daniel C, Becker JU. Comparison of different normalization strategies for the analysis of glomerular microRNAs in IgA nephropathy. *Sci Rep.* 2016;6:31992.

15. Kilens S, Meistermann D, Moreno D, et al. Parallel derivation of isogenic human primed and naive induced pluripotent stem cells. *Nat Commun.* 2018;9:360.

16. Meijer van Putten, Mengarelli I, Guan K, et al. Ion channelopathies in human induced pluripotent stem cell derived cardiomyocytes: a dynamic clamp study with virtual IK1. *Front Physiol*. 2015;6:7.

17. Gima K, Rudy Y. Ionic current basis of electrocardiographic waveforms: a model study. *Circ Res.* 2002;90:889-96.

18. Zygmunt AC, Eddlestone GT, Thomas GP, et al. Larger late sodium conductance in M cells contributes to electrical heterogeneity in canine ventricle. *Am J Physiol Heart Circ Physiol*. 2001;281:H689-697.

19. Li GR, Feng J, Yue L, Carrier M. Transmural heterogeneity of action potentials and Ito1 in myocytes isolated from the human right ventricle. *Am J Physiol*. 1998;275:H369-377.

20. Luo CH, Rudy Y. A model of the ventricular cardiac action potential. Depolarization, repolarization, and their interaction. *Circ Res.* 1991;68:1501-26.

21. Al Sayed ZR, Canac R, Cimarosti B, Bonnard C, Gourraud JB, Hamamy H, Kayserili H, Girardeau A, Jouni M, Jacob N, Gaignerie A, Chariau C, David L, Forest V, Marionneau C, Charpentier F, Loussouarn G, Lamirault G, Reversade B, Zibara K, Lemarchand P, Gaborit N. Human model of IRX5 mutations reveals key role for this transcription factor in ventricular conduction. *Cardiovasc Res*. 2020;8:cvaa259.

22. Morita H, Kusano KF, Miura D, et al. Fragmented QRS as a marker of conduction abnormality and a predictor of prognosis of Brugada syndrome. *Circulation*. 2008;118:1697-1704.

Supplemental figures:

Letter to Editor with previous submission number (CTM2-2021-01-0184)



Figure S1. Electrocardiogram of all subjects from whom hiPSCs were derived

Leads V1 to V3 from electrocardiogram of Non-BrS and BrS1-6 patients. + and – signs refer to the presence and absence of a *SCN5A* alteration variant, respectively. Type 1 BrS characteristics are visible in all BrS ECG and conduction defect is visible in Non-BrS ECG (right branch block).





Figure S2: Electrophysiological characterization of N1722D-SCN5A-expressing COS-7 cells.

The effects of N1722D-SCN5A variant present in BrS1⁺ hiPSC-CMs were characterized using COS-7 cells transfected with plasmids expressing either wild type (WT-SCN5A) or N1722D-SCN5A cDNA.

A. Illustrative immunostainings of Na_v1.5 (green) in COS-7 cells transfected with WT *SCN5A* and N1722D-*SCN5A* cDNA, showing robust Na_v1.5 expression of the variant at the cell surface. Nuclei were stained with DAPI (blue). B. Representative whole-cell current recorded in COS-7 cells overexpressing wild type (WT) and N1722D-*SCN5A* cDNA (left panel; voltage protocol in inset). Mean peak I_{Na} current densities (pA/pF) vs. membrane potential (Vm) (right panel). ** p<0.01 vs. WT (Two-way ANOVA with Bonferroni post-hoc test). C. Peak I_{Na} current densities, measured at -20 mV (Tukey plot), unveiling a significant reduction, by about 2 folds, of I_{Na} in N1722D-*SCN5A* transfected-COS-7 cells. *** p<0.001 vs. control (*t*-test).

D. I_{Na} voltage-dependence of inactivation and activation. For inactivation, I_{Na} was normalized to maximum (I/Imax), and plotted as a function of the potential of conditioning pulse that preceded the test pulse to -20 mV (inactivation; inset: voltage protocol). For activation, GNa (i.e. $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na+ ions) was normalized to maximum, and plotted as a function of Vm, the potential of the test pulse I_{Na} voltage dependence of activation. GNa (as $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na+ ions) was normalized to maximum, and plotted as a function of Vm, the potential of the test pulse I_{Na} voltage dependence of activation. GNa (as $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na+ ions) was normalized to maximum, and plotted as a function of Vm, the potential of the test pulse (activation: same voltage protocol as in B). This analysis did not reveal any modification of steady-state activation and inactivation, as in BrS1⁺ hiPSC-CMs.





Figure S3. I_{Na} and $I_{Ca,L}$ steady-state activation and inactivation gating properties in BrS hiPSC-CMs as compared to controls.

A. Left: I_{Na} voltage-dependence of inactivation. I_{Na} was normalized to its maximum value, and plotted as a function of the potential of conditioning pulse that preceded the -20-mV test pulse (inset: voltage protocol).

Right: I_{Na} voltage-dependence of activation. GNa (as $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na⁺ ions) was normalized to its maximum value, and plotted as a function of the potential of the test pulse (Vm; inset: voltage protocol).

B. I_{Ca,L} voltage-dependence of activation and inactivation (inset: voltage protocol).



Figure S4:

Figure S4. Ventricular action potential (AP) parameters in BrS hiPSC-CMs as compared to controls.

A. Representative ventricular-like AP when paced at 700 ms cycle length and when artificial I_{K1} was injected (dynamic current-clamp). APs are defined as ventricular-like when $(APD_{30}-APD_{40})/(APD_{70}-APD_{80})>1.45$.

B. Maximum upstroke velocity (dV/dt_{max}) of ventricular-like APs (Tukey plot). Conditions as in A. *p<0.05 *vs.* control (Mann-Whitney test).

C. AP overshoot from ventricular-like hiPSC-CMs. Conditions as in A. * p<0.05; vs control (t-test).

The AP maximum upstroke velocity (dV/dt_{max}) and overshoot were reduced in hiPSC-CMs presenting a reduction in I_{Na} .

D. Ventricular-like AP duration (APD) at 30%, 50% and 90% of full repolarization, showing that consistent with the absence of QT duration modification in BrS patients' ECGs, no difference in AP duration was observed between BrS and Ctrl hiPSC-CMs. Conditions as in A.

E. Beating frequencies of investigated cell lines. Box plots presenting peak-to-peak durations between action potentials, averaged for all spontaneously recorded action potentials (p = ns; One-way Anova test)





Figure S5. Proportions of ventricular action potentials with or without EADs.

Percentage of ventricular-like hiPSC-CMs presenting at least 1 EAD, irrespective of the current-clamp conditions, for each investigated clone of each hiPSC line.

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Supplemental Tables:

Table S1. Patient description

Subject	Genetic variant	Clinical Phenotype
Ctrl1	E1	-
Ctrl2	-	-
Ctrl3	-	-
Ctrl4	-	
BrS1 ⁺	SCN5A (p.N1722D)	BrS
BrS2 ⁺	SCN5A (p.A665G-fsX16)	BrS
BrS3 ⁻	-	BrS
BrS4 ⁻	-	BrS
BrS5	RRAD (p.R211H)	BrS
BrS6 ⁻	-	BrS
Non-BrS	SCN5A (p.A665G-fsX16)	PCCD

Table S2. ECG patient characteristics.

ECGs were performed in leads D1, D2, V1, V2, V3 and recordings used the following parameters: 25 mm/s, 0.1 mV/mm.

RR interval duration (RR); S wave duration and amplitude (S); P wave duration (P); PR interval duration (PR); QRS duration (QRS); QT peak interval (QTp); QT end interval (QTe); Tpeak-to -Tend interval (TPE); J wave amplitude (J); Early Repolarization Pattern (ERP); Fragmented QRS according to Morita H et al.²² (Frag); Concave aspect of the ST segment elevation (Concave aspect); Yes (y); No (n).

		D1			D2				V1		V2		V3		
	RR ms	S ms	S mm	Pms	PR ms	QRS ms	QTp ms	QTe ms	TPE ms	Jmm	TPE ms	J mm	TPE ms	Jmm	TPE ms
Ctrl	837.8	40.5	2.4	101.4	168.9	81.1	290.5	351.4	60.8	0.3		0.7	81.1	0.7	81.1
BrS1+	709.5	47.3	2.2	128.4	229.7	121.6	297.3	371.6	74.3	4.6	60.8	4.6	74.3	0.5	47.3
BrS2+	959.7	67.1	5.7	114.1	221.5	107.4	295.3	375.8	80.5	3.0	100.7	4.2	107.4	1.8	100.7
BrS3-	797.3			108.1	189.2	121.6	277.0	378.4	101.4	2.2	108.1	1.7	195.9	0.5	121.6
BrS4-	798.7	40.3	1.2	94.0	174.5	94.0	275.2	349.0	73.8	b.8	60.4	2.9	47.0	2.3	80.5
BrS5-	831.1	27.0	1.0	94.6	162.2	81.1	297.3	364.9	67.6	1.9	54.1	4.7	101.4	2.4	128.4
BrS6-	763.2	96.5	3.1	105.3	166.7	131.6	289.5	377.2	87.7	3.1	122.8	1920	149.1	-0.9	105.3
Non-BrS	771.8	13.4	0.7	87.2	187.9	100.7	255.0	328.9	73.8	0.3	67.1	1.0	60.4	1.8	80.5

	ERP	Frag	N	egative T wa	ve	0	concave aspec	ct
	y/n		V1	V2	V3	V1	V2	V3
Ctrl	n	n	n	n	n	n	n	n
BrS1+	n	n	Y	У	n	У	У	n
BrS2+	n	n	Y	У	n	У	У	n
BrS3-	У	n	Y	У	n	У	n	n
BrS4-	n	n	Y	n	n	n	n	n
BrS5-	n	n	Y	У	У	У	У	n
BrS6-	n	n	Y	У	n	n	n	n
Non-BrS	n	n	Y	n	n	n	n	n

Table S3. TLDA probe references and	d corresponding genes
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gene symbol	nrotein symbol	Gene name	Assay ID	Category
ADCCO		ATD binding months with family C (CTTD (MDD)) months 0	H-010027611	Datassium (K ¹) shamed
ABCC8	SUR1	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	Hs01093761_m1	Potassium (K [°]) channel
ABCC9	SUR2	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	Hs00245832_m1	Potassium (K [*]) channel
ATP1A3	Na/K-ATPase a3	ATPase, Na+/K+ transporting, alpha 3 polypeptide	Hs00958036_m1	Na ⁺ /K ⁺ ATPase
ATP1B1	Na/K-ATPase b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	Hs00426868 g1	Na ⁺ /K ⁺ ATPase
ATP2A2	SERCA2	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	Hs00544877 m1	Calcium (Ca ⁺⁺) regulator
470342	SERCAR	ATPace Could transporting, uniquitour	Hc00103000 m1	Calcium (Ca ⁺⁺) regulator
ATF2A5	SERCAS	ATPase, carr transporting, doiquitous	1500193090_111	calcium (ca) regulator
ATP2B4	Ca ATPase4	A IPase, Ca++ transporting, plasma membrane 4	HS00608066_m1	Calcium (Ca) regulator
CACNA1C	Cav1.2	calcium channel, voltage-dependent, L type, alpha 1C subunit	Hs00167681_m1	Calcium (Ca++) channel
CACNA1D	Cav1.3	calcium channel, voltage-dependent, L type, alpha 1D subunit	Hs00167753_m1	Calcium (Ca++) channel
CACNA1G	Cav3.1	calcium channel, voltage-dependent, alpha 1G subunit	Hs00367969 m1	Calcium (Ca++) channel
CACNA1H	Cav3.2	calcium channel, voltage-dependent, alpha 1H subunit	Hs01103527 m1	Calcium (Ca++) channel
CACNA3D1	Cava2d1	calcium channel, voltage dependent, alpha 1/1 subunit	Hc00084856 m1	Calcium (Caul) channel
CACNAZDI	cavazui	calcium chamer, vortage-dependent, alpha 2/deita subunit 1	1500584850_111	calcium (ca++) channel
CACNA2D2	Cava2d2	calcium channel, voltage-dependent, alpha 2/delta subunit 2	Hs01021049_m1	Calcium (Ca++) channel
CACNB2	Cavb2	calcium channel, voltage-dependent, beta 2 subunit	Hs01100744_m1	Calcium (Ca++) channel
CALM1	CALM1	calmodulin 1 (phosphorylase kinase, delta)	Hs00300085_s1	Calcium (Ca ⁺⁺) regulator
CALM3	CALM3	calmodulin 3 (phosphorylase kinase, delta)	Hs00968732_g1	Calcium (Ca ⁺⁺) regulator
CASO2	CASO2	calsequestrin 2 (cardiac muscle)	Hs00154286 m1	Calcium (Ca ⁺⁺) regulator
GIA1	Cv42	ran junction protoin, alpha 1, 42kDa (connovin 42)	Hc00748445_c1	Connovin
GIAI	CX45	gap junction protein, aipha 1, 45kba (connexin 45)	1500748445_51	Connexin
GJA5	Cx40	gap junction protein, alpha 5, 40kba (connexin 40)	Hs00270952_m1	Connexin
GJA7	Cx45	gap junction protein, alpha 7, 45kDa (connexin 45)	Hs00271416_s1	Connexin
GJD3	Cx30.2	gap Junction Protein, delta 3, 31.9kDa	Hs00987388_s1	Connexin
HCN1	HCN1	hyperpolarization activated cyclic nucleotide-gated potassium channel 1	Hs01085412_m1	Cation channel
HCN2	HCN2	hyperpolarization activated cyclic nucleotide-gated notassium channel 2	Hs00606903 m1	Cation channel
HCN2	HCND	hyperpalarization activated cyclic nucleotide gated potaccium channel 3	Hc00380018 m1	Cation channel
HCN3		hyperpolarization activated cyclic nucleotide-gated potassium channel 5	1500580018_111	
ncN4	nun4	hyperpolarization activated cyclic nucleotide-gated potassium channel 4	nsuu975492_m1	Cation channel
IT'PR1	ITPR1	Inositol 1,4,5-triphosphate receptor, type 1	HsU0181881_m1	Calcium (Ca ^{**}) regulator
ITPR3	ITPR3	inositol 1,4,5-triphosphate receptor, type 3	Hs00609908_m1	Calcium (Ca ⁺⁺) regulator
KCNA2	Kv1.2	potassium voltage-gated channel, shaker-related subfamily, member 2	Hs00270656_s1	Potassium (K ⁺) channel
KCNA4	Kv1.4	potassium voltage-gated channel, shaker-related subfamily, member 4	Hs00937357 s1	Potassium (K ⁺) channel
KCNA5	Kv1 5	notassium voltage-gated channel, shakar-related subfamily, member 5	Hs00969279 s1	Potassium (K ⁺) channel
KCNAZ	Ku1 7	potassium voltage gated channel, shaker related sublahiny, melliber 5	H-002610101	Detective (V ⁺) -
KCNA/	NV1./	porassium vortage-gareo channel, snaker-related subfamily, member 7	UP00201012_W1	rotassium (K.) channel
KCNAB2	Kvb2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	Hs00186308_m1	Potassium (K [*]) channel
KCNAB3	Kvb3	potassium voltage-gated channel, shaker-related subfamily, beta member 3	Hs01085073_m1	Potassium (K ⁺) channel
KCNB1	Kv2.1	potassium voltage-gated channel, Shab-related subfamily, member 1	Hs00270657_m1	Potassium (K ⁺) channel
KCNC4	Kv3.4	potassium voltage-gated channel, Shaw-related subfamily, member 4	Hs00428198 m ¹	Potassium (K ⁺) channel
KCND2	Kv4 2	notassium voltage-gated channel. Shal-related subfamily, member 2	Hs01054873 m1	Potassium (K ⁺) channel
KCND2	Ku4 2	potassium voltage-gated channel, Shal-related subianily, member 2	H-00E42E071	Detessive (k) -
NCIND 3	r.v4.3	porassium vorrage-gareo channel, shai-related subfamily, member 3	ns0054259/_M1	rotassium (K.) channel
KCNE1	MinK	potassium voltage-gated channel, Isk-related family, member 1	Hs00264799_s1	Potassium (K [*]) channel
KCNE1L	MIRP4	potassium voltage-gated channel, Isk-related family, member 1-like	Hs01085745_s1	Potassium (K [*]) channel
KCNE2	MIRP1	potassium voltage-gated channel, Isk-related family, member 2	Hs00270822 s1	Potassium (K ⁺) channel
KCNE3	MIRP2	notassium voltage-gated channel. Isk-related family, member 3	Hs01921543_s1	Potassium (K ⁺) channel
KCNEA	MIRD3	potassium voltage-gated channel, isk-related family, member 4	Hc01851577 c1	Potassium (K ⁺) channel
KCNE4		potassium voltage-gated channel, isk-related family, member 4	1501851577_51	
KCNHZ	hERG	potassium voltage-gated channel, subfamily H (eag-related), member 2	Hs04234270_g1	Potassium (K [°]) channel
KCNIP2	KChIP2	Kv channel interacting protein 2	Hs01552688_g1	Potassium (K [*]) channel
KCNJ11	Kir6.2	potassium inwardly-rectifying channel, subfamily J, member 11	Hs00265026_s1	Potassium (K ⁺) channel
KCNJ2	Kir2.1	potassium inwardly-rectifying channel, subfamily J, member 2	Hs01876357_s1	Potassium (K ⁺) channel
KCNJ3	Kir3.1	notassium inwardly-rectifying channel, subfamily 1, member 3	Hs04334861 s1	Potassium (K ⁺) channel
KCNIA	Kir2 3	potassium inwardly-rectifying channel, subfamily I, member 4	Hc00705379 c1	Potassium (K ⁺) channel
KCNJ4	NIT 2.5	potassium inwardiy-rectifying channel, subfamily J, member 4	HS00705379_51	Potassium (K) channel
KCNJ5	KIr3.4	potassium inwardiy-rectifying channel, subtamily J, member 5	HS00168476_m1	Potassium (K.) channel
KCNJ8	Kir6.1	potassium inwardly-rectifying channel, subfamily J, member 8	Hs00958961_m1	Potassium (K [*]) channel
KCNK1	TWIK1	potassium channel, subfamily K, member 1	Hs00158428_m1	Potassium (K ⁺) channel
KCNK3	TASK1	potassium channel, subfamily K, member 3	Hs00605529 m1	Potassium (K ⁺) channel
KCNK5	TASK2	notassium channel, subfamily K, member 5	Hs00186652 m1	Potassium (K ⁺) channel
KCNO1	Kyl OT1	potassium voltage-gated channel. KOT-like subfamily, member 1	Hc00923522 m1	Potassium (K ⁺) channel
KCNQI	AND AND	potassium vortage-gated channel, kor-like sublanniy, member 1	1500923322_111	rotassium (k.) channer
NPPA	ANP	natriuretic peptide precursor A	HS00383230_g1	Signaling molecule
NPPB	BNP	natriuretic peptide precursor B	Hs01057466_g1	Signaling molecule
PLN	PLN	phospholamban	Hs01848144_s1	Calcium (Ca ⁺⁺) regulator
PPP3CA	CAM-PRP	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	Hs00174223 m1	Calcium (Ca ⁺⁺) regulator
RYR2	RYR2	rvanodine recentor 2 (cardiac)		Calcium (Ca ⁺⁺) regulator
SCN10A	Nav1 8	sodium channel voltage-gated type X alpha	Hs01045137 m1	Sodium (Na ⁺) channel
SCIVIDA	INdV1.0	soulum channel, voltage-gated, type X, alpha	1501043137_111	
JCN1D	INGVD1	sourum channel, voltage-gated, type I, Deta	1202201023_M1	socium (Na.) channel
SCN2B	wavb2	sodium channel, voltage-gated, type II, beta	HSUU394952_m1	Socium (Na) channel
SCN3A	Nav1.3	sodium channel, voltage-gated, type III, alpha	Hs00366913_m1	Sodium (Na [*]) channel
SCN3B	Navb3	sodium channel, voltage-gated, type III, beta	Hs01024483_m1	Sodium (Na ⁺) channel
SCN4A	Nav1.4	sodium channel, voltage-gated, type IV, alpha	Hs01109480_m1	Sodium (Na ⁺) channel
SCN4B	Navb4	sodium channel, voltage-gated, type IV, beta	Hs03681025 m1	Sodium (Na ⁺) channel
SCN5A	Nav1.5	sodium channel, voltage-gated, type V, alpha (long OT syndrome 2)	Hs00165693 m1	Sodium (Na ⁺) channel
SCN7A	Nav2 1	codium channel voltage gated, type v, apha (long of syndrome 3)	Hc00161546 m1	Sodium (Na ⁺) channel
		sources chaines, voltage-gateu, type vii, aipila		sources (na) channel
SUNSA	INAV1./	sourum channel, voitage-gated, type IX, alpha	msuu161567_m1	Soulum (Na) channel
SLC8A1	NCX1	solute carrier family 8 (sodium/calcium exchanger), member 1	HsU1062258_m1	Calcium (Ca ^{**}) regulator
ANK2	ANKB	ankyrin 2	Hs00153998_m1	Cytoskeletal protein
GATA3	GATA3	GATA Binding Protein 3	Hs00231122_m1	Transcription factor
GATA4	GATA4	GATA Binding Protein 4	Hs00171403 m1	Transcription factor
GATA5	GATA5	GATA Binding Protein 5	Hs00388359 m1	Transcription factor
CATAG	CATAS	GATA Rinding Brotoin 6	H=00222018 m1	Transcription factor
GAIAO	GATAU		1300232010_001	manscription ractor
HEYZ	HEY2	Hes Kelated Family BHLH Transcription Factor With YRPW Motif 2	HSUU232622_m1	ranscription factor
IRX3	IRX3	iroquois homeobox 3	Hs01124217_g1	Transcription factor
IRX4	IRX4	iroquois homeobox 4	Hs00212560_m1	Transcription factor
IRX5	IRX5	iroquois homeobox 5	Hs04334749_m1	Transcription factor
твх2	TBX2	T-Box Transcription Factor 2	Hs00911929 m1	Transcription factor
TRV2	TRV2	T Box Transcription Factor 2	Hc0010E612 m1	Transcription factor
10/0		T Dev Tenenerinking Factor 5	11500153012_001	Transcription ractor
18X5	18X2	I-BOX Transcription Factor 5	HSUU361155_m1	ranscription factor
NKX2.5	NKX2-5	NK2 Homeobox 5	Hs00231763_m1	Transcription factor
TNNI3	TNNI3	Troponin I3, Cardiac Type	Hs00165957_m1	Cytoskeletal protein
TNNT2	TNNT2	Troponin T2, Cardiac Type	Hs00943911_m1	Cytoskeletal protein
МҮН6	MYH6	Myosin Heavy Chain 6	Hs01101425 m1	Cytoskeletal protein
MVH7	MVH7	Muosin Heavy Chain 7	Hc01110632 m1	Cutoskeletal protein
		PRAD, Des Deleted Churcheric 1, 1111 - 1, 10, 11, 21, 11, 11, 11, 11, 11, 11, 11, 11	11501110052_001	Cytoskeletal protein
KKAD	KAD	KKAD, Kas Kelated Glycolysis Inhibitor And Calcium Channel Regulator	HSUU188163_m1	GIP-binding protein
CLASP2	CLASP2	Cytoplasmic Linker Associated Protein 2	Hs00380556_m1	Cytoskeletal protein
GPD1L	GPD1L	Glycerol-3-Phosphate Dehydrogenase 1 Like	Hs00380518_m1	Enzyme
MYL7	MLC2A	Myosin Light Chain 7	Hs01085598 g1	Cytoskeletal protein
MYL2	MLC2V	Myosin Light Chain 2	Hs00166405 m1	Cytoskeletal protein
100		Eukapuatia 19a rBNA	H=00000001 c1	andoganous
103	0.244	Date 2 Missa elekulia	1200102042	endogenous control
R5W	B2M	Beta-2-Microglobulin	HSUU187842_m1	enaogenous control
АСТВ	АСТВ	actin beta	Hs99999903_m1	endogenous control
RPL13A	RPL13A	Ribosomal Protein L13a	Hs04194366 g1	endogenous control

Table S4. Mean value \pm SEM of I_{Na} and $I_{Ca,L}$ activation and inactivation kinetics parameters in the different hiPSC-CMs lines. V1/2 and K represent voltage of half-maximum (in)activation and slope factor, respectively.

		0	Ctrl	BrS1 ⁺	BrS2 ⁺	BrS3-	BrS4 ⁻	BrS5	BrS6	Non- BrS
		V _{1/2}	-33.22	-32.54	-33.55	-32.91	-33.79	-33.58	-33.22	-33.21
	Activation	(mV)	± 0.3	± 0.2	± 0.6	± 0.2	± 0.5	± 0.2	± 0.6	± 0.2
		K	6.244	6.122	6.553	6.369	6.372	5.689	6.935	5.947
I _{Na}		(mV)	± 0.2	± 0.2	± 0.4	± 0.1	± 0.4	± 0.1	± 0.4	± 0.1
	Inactivation	V _{1/2}	-87.17	-86.4	-88.13	-85.51	-87.53	-85.91	-86.92	-88.12
		(mV)	± 0.2	± 0.4	± 0.6	± 0.2	± 0.3	± 0.2	± 0.2	± 0.4
		K	-5.691	-5.448	-6.381	-6.262	-6.029	-6.004	-5.609	-5.444
		(mV)	± 0.1	± 0.3	± 0.5	± 0.2	± 0.2	± 0.1	± 0.1	± 0.4
		$V_{1/2}$	-16.62	-18.47	-1 9.41	-17.79	-17.21	-18.54	-1 9.71	-18.49
	A	(mV)	± 0.3	± 0.3	± 1.1	± 0.3	± 1.1	± 0.3	± 0.4	± 0.6
	Activation	K	7.006	6.064	7.522	6.86	6.777	6.265	6.322	6.68
		(mV)	± 0.2	± 0.2	± 0.8	± 0.2	± 0.8	± 0.2	± 0.3	± 0.4
I _{Ca,L}		V _{1/2}	-46.92	-44.85	-49.38	-47.49	-49.69	-49.69	-44.8	-46.1
	Inactivation ·	(mV)	± 0.2	± 0.2	± 0.5	± 0.2	± 0.5	± 0.2	± 0.4	± 0.3
		K	-7.429	-6.406	-6.843	-7.251	-8.087	-7.167	-7.257	-6.581
		(mV)	± 0.2	± 0.2	± 0.4	± 0.2	± 0.4	± 0.2	± 0.4	± 0.3

Table S5. Correlation between ECG parameters and the corresponding hiPSC-CM sodium currents.

		Correlation coe	fficient r _s of Spearman
		Late I _{Na}	Peak I _{Na}
	RR (ms)	0.48	-0.07
D1	S duration (ms)	-0.55	0.00
	S amplitude (mm)	-0.10	0.26
	P (ms)	-0.47	0.01
D2	PR (ms)	-0.63	0.20
	QRS (ms)	-0.55	-0.31
	QTp (ms)	-0.40	-0.14
	QTe (ms)	-0.32	-0.14
	TPE (ms)	-0.07	-0.14
V1	J amplitude (mm)	-0.81 *	0.02
	TPE (ms)	0.11	-0.14
V2	J amplitude (mm)	-0.86 *	0.14
	TPE (ms)	-0.33	-0.26
V3	J amplitude (mm)	0.23	0.19
	TPE (ms)	-0.23	-0.31

Each ECG parameter was tested for its correlation with either $I_{Na,L}$ or peak I_{Na} density from the corresponding hiPSC-CMs. While the J point elevation correlated significantly with $I_{Na,L}$ density, it did not correlate with hiPSC-CM peak I_{Na} density. Statistical significance threshold of r_s was p < 0.05 (*).

Response to decision letter

Responses to Editor

Editor: Please merge Supplemental Figures 2 and 3 as ONE and add it into the text as Figure 4.

<u>Response</u>: Dear Editor, we have performed the figure modification accordingly, however, we numbered this new figure as Figure 1 and not Figure 4, as it is now the first described figure in the Letter.

Responses to Reviewer

<u>Reviewer</u>: Al Sayed and colleagues used a 2-dimentional patient-derived iPSC-CMs to collate data that reveal BrS molecular alterations and a story consequently, which is indeed important in understating the molecular mechanisms of the disease. Particularly, the genetic characterisation adds to the understanding. I would recommend the following minor comments:

<u>Reviewer</u>: 1) Define GS-458967 at first use.

<u>Response</u>: We thank the Reviewer for pointing out this mistake, and clarified at the first use of GS-458967 that its full name is 6-(4-(Trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine and that selectively blocks late sodium current. We added this explanation on page 3, paragraph 3, of the Letter.

<u>Reviewer</u>: 2) Increased triggered activity is associated with beating frequency. The Baseline-like arrhythmic events and average number of cells with increase beating frequency after baseline could be demonstrated.

<u>Response</u>: We thank the Reviewer for this very relevant comment. Upon request, we performed a comparative analysis of spontaneous beating frequencies of hiPSC-CMs from all studied lines. We did not observe any statistical difference between the BrS lines and the Ctrl or Non-BrS lines (Figure S4E; p = ns; One-way Anova test). Therefore, in opposition to what we observed for the EAD occurrence (higher in BrS lines as compared to Ctrl and Non-BrS), abnormal beating frequency was not a common phenotype of the BrS lines. Therefore, the EAD phenotype could not be explained by an altered spontaneous beating frequency in BrS lines as compared to Ctrl and Non-BrS lines. This was added to the Letter on page 3, paragraph 2, and the new figure was added to the supplemental material (Figure S4E).

<u>Reviewer</u>: 3) The study nature, depth and statistical power of the samples may be insufficient to summit common cellular phenotypic singularities in feature as the basis of specific BrS ECG morphogenesis. I would suggest adjusting (playing down) the hypothesis to spot-on match the study findings and conclusion. BrS has been referred to as an overlap syndrome (channelopathy vs cardiomyopathy), and can result from a numerous diverse underlying pathophysiological mechanisms.

Response: We understand the Reviewer's concern and we have changed the conclusion accordingly: "In conclusion, in the present study, a particular cellular electrophysiological phenotype common to six out of six BrS hiPSC-CM lines with various genetic backgrounds has been unveiled." This change has been made on page 4, paragraph 2, of the Letter.

<u>Reviewer</u>: 4) The unique signature of gene expression and trigger potentials (EAD) of each hiPSC-CMs of the three clones selected per patient needs comment and could be added as a figure and in few lines to the body of the letter. **<u>Response</u>**: We thank the Reviewer for his suggestions. Concerning the transcriptomic data, to follow the Reviewer's comment, we annotated Figure 1E and Figure 2A highlighting all clones of each hiPSC line and changed the Figure legends accordingly. This shows that the clone-to-clone heterogeneity is lower than the cell line-to-cell line heterogeneity. Regarding the investigation of BrS hiPSC-CM clone–specific signature, unfortunately, the study was not designed nor powered to answer this specific question. In addition, the low number of patient cell lines available in this study might not have been sufficient to reliably describe clone-to-clone heterogeneity. Therefore, we respectfully believe that this is beyond the scope of the present letter. However, we thank the Reviewer for his comment, as it may trigger a future project aiming at unveiling heterogeneity among various BrS hiPSC lines, including at the transcriptomic level, with a potential link to the patients' phenotype.

Regarding the data on EADs, we displayed the percentage of ventricular cells with and without EADs, for each investigated clone, of each hiPSC line, showing good reproducibility of the data (both for the control and for the BrS cell lines). We added this figure to the supplementary material, as Figure S5.

<u>Reviewer</u>: 5) It would be difficult to some extent for non-specialist to follow that "Furthermore, INa biophysical properties were not modified in BrS hiPSC-CMs (Figure S5A; Table S4)" supports the argument that precedes it. This could be as a result of overly condensed writing. The authors should find a way to make the sentence more congruent to what is being said before it.

<u>Response</u>: The Reviewer has a point and we apologize for the lack of clarity, due indeed to an oversimplification of the original sentences. By "INa biophysical properties", we meant the activation and inactivation kinetics of the sodium channels. We modified the paragraph to make it clearer and more meaningful: "Concordantly, reduction in INa density was detected in these same lines (Figure 2F-2H). This confirmed previous results, for BrS5+, and regarding BrS1+, which carries an SCN5A rare variant, the reduction was confirmed using conventional transfection in COS-7 cells of this variant (Figure S2). Furthermore, the steady-state activation and inactivation gating properties were not modified in BrS hiPSC-CMs (Figure S3A; Table S4)." (See page 2, paragraph 4).

<u>Reviewer</u>: 6) Refer to "thereby, high EAD occurrence in ventricular-like hiPSC-CMs seemed to be associated with BrS, and not with the presence of SCN5A variant" if ventricular-like hiPSC-CMs from patients with SCN5A variants had no AP triggering activity at all, it should be made clear.

<u>Response</u>: We realize from this Reviewer's comment, that this sentence was misleading. To sum up the findings, (1) triggered activities were observed in all studied hiPSC-CM lines, but with very different occurrences; (2) all 6 BrS-derived cell lines, both the ones that carry variants in SCN5A and the ones that do not, displayed high occurrences of EADs (in 39% to 70% of the ventricular hiPSC-CMs) while (3) EAD occurrence was much lower in ventricular hiPSC-CMs of the Control cell lines (4%) and (4) EAD occurrence was also much lower in the "Non-BrS" cell line (4.7%), which carries a variant in SCN5A but is not affected by BrS. Using Fisher's exact test we confirmed the statistical significance of our findings. Therefore, the occurrence level of these triggered activities seemed to be associated to the presence of a BrS phenotype in the investigated cell lines, but not to the presence of a variant in SCN5A. We modified accordingly this part of the results on page 3, paragraph 2, of the Letter.

<u>Reviewer</u>: 7) The phenotype investigated may be a product of complex interaction of multiple cell types, genetics, and the environment, and therefore iPSC-CMs may be an unsuitable model. Whether or not this view holds for the model could be expanded on in relation to the findings.

<u>Response</u>: The Reviewer raises a critical point that should indeed be acknowledged in the study. We agree with the Reviewer that the use of hiPSC-CMs in the actual setting may not fully model the complex pathophysiology of BrS. First, as pointed out by the Reviewer the cardiac differentiation of hiPSCs generates a heterogeneous population of cardiac cell types that may all be involved in the BrS

pathophysiology. In the present study, we focused on the ventricular cell type however, further studies will have to investigate the potential involvement of the other cell types in the disease phenotype. Second, the generated cells are not organized in layers or compartments and do not recapitulate the specific cell-to-cell interactions that take place in the cardiac tissue. Altogether, this, most probably, currently limits the complete understanding of the BrS mechanism. The implementation of emerging phenotypic technologies, such as single-cell transcriptomics and cardiac tissue engineering, should undoubtedly further expand the power of investigating BrS with the hiPSC technology. We added these notions to the conclusion of the Letter, on page 4, paragraph 2.

for Review Only

A consistent arrhythmogenic trait in Brugada syndrome cellular phenotype

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Dear Editor,

Brugada syndrome (BrS) is an inherited arrhythmic disease predisposing to sudden cardiac death (SCD), characterized by a typical electrocardiogram pattern that includes a J point elevation with a coved type ST segment.¹ BrS is a complex genetic disease in which ~20% of patients carry rare variants in *SCN5A* gene whereas the others remain genetically unresolved.² Despite this genetic complexity, we hypothesize that a common cellular phenotypic trait is at the root of this specific BrS ECG pattern. In this study, we identified a phenotype that is common to human induced pluripotent stem cell-derived ventricular cardiomyocytes (hiPSC-CMs) generated from six Brugada patients with different genetic backgrounds. Our results unmasked a cellular arrhythmogenic phenotype combining gene expression and electrical abnormalities, including an increase in late sodium current.

Six patients affected by type I BrS (BrS1-6; Figure S1; Table S1; Table S2) with a familial history of SCD or syncope were selected, among whom two carry *SCN5A* variants (marked with a ⁺ symbol). An additional individual, not affected by BrS (Non-BrS), carrying the same *SCN5A* variant as BrS2⁺, was also recruited, as well as four control (Ctrl) subjects. Somatic cells from all studied subjects were reprogrammed into hiPSC lines and differentiated into cardiomyocytes (Figure 1).

Transcriptional expression profiling identified 133 differentially expressed genes in BrS hiPSC-CMs (Figure 2A). Gene Set Enrichment Analyses showed that transcripts of transmembrane transporters and channels were significantly overrepresented (Figure 2B), including genes encoding sodium, calcium, and potassium channels (Figure 2C). High-throughput real-time RT-PCR,³ on 96 genes related to cardiac electrical function (Table S3) identified 13 differentially expressed genes in BrS, in comparison to Ctrl and Non-BrS hiPSC-CMs (Figure 2D). Importantly, the expression of *SCN5A*, the main BrS culprit gene identified to-date,⁴ remained unchanged, excluding *SCN5A* expression levels as a hallmark for BrS hiPSC-CM phenotype. Conversely, calcium and sodium transporters, playing important roles in membrane depolarization, were differentially expressed. Comparative analysis of hiPSC-CM electrophysiological functions investigated whether these modifications were a consistent trait of BrS phenotype at the cellular level.

Whereas decrease in sodium current is considered as the most frequently associated electrical alteration in BrS pathophysiology,^{5,6} protein expression of Na_v1.5, encoded by *SCN5A*, was decreased in only 2 BrS, and the Non-BrS lines (Figure 2E). Concordantly, reduction in I_{Na} density was detected in these same lines (Figure 2F-2H). This confirmed previous results, for BrS5⁺,⁷ and regarding BrS1⁺, which carries an *SCN5A* rare variant, the reduction was confirmed using conventional transfection in COS-7 cells of this variant (Figure S2). Furthermore, the steady-state activation and inactivation gating properties were not modified in BrS hiPSC-CMs (Figure S3A; Table S4). Therefore, I_{Na} reduction is not a common trait of BrS hiPSC-CMs and appears to be solely associated with the presence of variants affecting *SCN5A* expression or function.

Similarly, reduction in $I_{Ca,L}$ channel protein expression and current density were not a common trait of BrS hiPSC-CMs (Figure 2I-2L, Figure S3B; Table S4).

Global cellular electrophysiological phenotype was then evaluated with action potential (AP) recordings, but no AP basal parameters specifically segregated BrS hiPSC-CMs, and spontaneous beating frequencies did not differ between all cell lines (Figure S4). Noteworthy, ventricular-like AP analysis revealed an arrhythmic phenotype present mostly in BrS hiPSC-CMs, irrespective of their genetic background (Figure 3A). Early afterdepolarizations (EADs) were observed in 39% to 70% of all six BrS ventricular-like hiPSC-CMs versus only in 4% and 4.7% of Ctrl and Non-BrS hiPSC-CMs, respectively (Figure 3B, S5). Thereby, the high EAD occurrence in ventricular-like hiPSC-CMs was associated with the presence of a BrS phenotype in the investigated cell lines, but not to the presence of a variant in *SCN5A*.

The occurrence of EADs may be linked to an abnormally high density of depolarizing late sodium current ($I_{Na,L}$) during APs repolarizing phase.⁸ Accordingly, BrS hiPSC-CMs presented with a higher density of $I_{Na,L}$ as compared to Ctrl and Non-BrS hiPSC-CMs (Figure 3C,3D). Moreover, an increase in $I_{Na,L}$ density was observed only in 6% and 12% of Ctrl and Non-BrS hiPSC-CMs respectively, in accordance with their low EAD occurrence, whereas increased $I_{Na,L}$ density was present in 50% to 85% of all BrS ventricular-like hiPSC-CMs, reminiscent of the high EAD occurrence (Figure 3B,3E). We then superfused ventricular-like BrS hiPSC-CMs during AP recording with GS-458967 (6-(4-(Trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine, which selectively blocks late sodium current),⁹ causing full inhibition of $I_{Na,L}$ (Figure 3F), and found abolishment of EADs (Figure 3G,3H) and reduced APD90 dispersion (Figure 3I). Altogether, these data strongly suggested that the abnormal increase of $I_{Na,L}$ in BrS hiPSC-CMs is responsible for EADs.

Further strengthening the role of the $I_{Na,L}$ in the electrical cellular phenotype of BrS, when each ECG parameter was tested for its correlation with either $I_{Na,L}$ or I_{Na} measured densities, only $I_{Na,L}$ density correlated significantly with one sole parameter, *i.e.*, the J point elevation (Table S5).

To challenge the pathophysiological relevance of the ion current alterations identified in Non-BrS and BrS2⁺ hiPSC-CMs, we applied them to a mathematical human electrogram model, that allows visualizing transmural-like electrogram with a QRS-like complex, a ST-like segment, and a T-like wave (Figure 4A).¹⁰ First, in accordance with BrS2⁺ patient's ECG, applying the alterations observed in peak I_{Na} , $I_{Ca,L}$ and in $I_{Na,L}$ in BrS2⁺ hiPSC-CMs was sufficient to induce prolongation of the QRS-like complex, ST-like segment elevation and widening, and T-like wave inversion (Figure 4B). Then, sequential correction of each altered current in BrS2⁺ hiPSC-CMs was made (BrS2⁺_{corrected}). Correction of I_{Na} density led to QRS-like complex normalization; correction of $I_{Ca,L}$ density shortened the duration of the ST-like segment elevation and normalized the T-like wave orientation; and correction of $I_{Na,L}$ density led to reduction of ST-like segment amplitude towards normalization (Figure 4C, left to right). Overall, these

results strongly suggest that depolarizing currents alterations can impact a multi-cellular electrogram model, mimicking BrS ECG phenotype.

In conclusion, in the present study, a particular cellular electrophysiological phenotype common to six out of six BrS hiPSC-CM lines with various genetic backgrounds has been unveiled. We showed that high EAD occurrence associates with an abnormal increase of I_{Na,L} in all investigated BrS cell lines, and correlates with the corresponding patients' J point elevation on ECG. We focused on the ventricular cell type, at a single-cell level. Implementation of emerging phenotypic technologies, such as singlecell transcriptomics and cardiac tissue engineering, will allow investigation of the potential involvement of the other cardiac cell types in the disease phenotype and the role of specific cell-tocell interactions. Altogether, the obtained results open perspectives to better understand the ventricular arrhythmia occurrence in BrS and to identify a dedicated therapeutic approach to prevent the risk of SCD.

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Conflict of Interest

None.

Data availability statement.

In accordance with the "DFG Guidelines on the Handling of Research Data", the authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. The data set will be archived for at least 10 years after publication.

References

1. Priori SG, Wilde AA, Horie M, et al. HRS/EHRA/APHRS Expert Consensus Statement on the Diagnosis and Management of Patients with Inherited Primary Arrhythmia Syndromes. *Heart Rhythm*. 2013;10:1932-63.

2. Gourraud J-B, Barc J, Thollet A, et al. The Brugada Syndrome: A Rare Arrhythmia Disorder with Complex Inheritance. *Front Cardiovasc Med*. 2016;3:9.

3. Al Sayed ZR, Canac R, Cimarosti B, Bonnard C, Gourraud JB, Hamamy H, Kayserili H, Girardeau A, Jouni M, Jacob N, Gaignerie A, Chariau C, David L, Forest V, Marionneau C, Charpentier F, Loussouarn G, Lamirault G, Reversade B, Zibara K, Lemarchand P, Gaborit N. Human model of IRX5 mutations reveals key role for this transcription factor in ventricular conduction. *Cardiovasc Res.* 2020;8:259.

4. Kapplinger JD, Tester DJ, Alders M, et al. An international compendium of mutations in the SCN5A-encoded cardiac sodium channel in patients referred for Brugada syndrome genetic testing. *Heart Rhythm*. 2010;7:33-46.

5. Yan GX, Antzelevitch C. Cellular basis for the Brugada syndrome and other mechanisms of arrhythmogenesis associated with ST-segment elevation. *Circulation*. 1999;100:1660-6.

6. Meregalli PG, Wilde AA, Tan HL. Pathophysiological mechanisms of Brugada syndrome: depolarization disorder, repolarization disorder, or more? *Cardiovasc Res.* 2005;67:367-78.

7. Belbachir N, Portero V, Al Sayed ZR, Gourraud JB, Dilasser F, Jesel L, Guo H, Wu H, Gaborit N, Guilluy C, Girardeau A, Bonnaud S, Simonet F, Karakachoff M, Pattier S, Scott C, Burel S, Marionneau C, Chariau C, Gaignerie A, David L, Genin E, Deleuze JF, Dina C, Sauzeau V, Loirand G, Baró I, Schott JJ, Probst V, Wu JC, Redon R, Charpentier F, Le Scouarnec S. RRAD mutation causes electrical and cytoskeletal defects in cardiomyocytes derived from a familial case of Brugada syndrome. *Eur Heart J*. 2019 Oct 1;40(37):3081-3094.

8. Shryock JC, Song Y, Rajamani S, et al. The arrhythmogenic consequences of increasing late INa in the cardiomyocyte. *Cardiovasc Res.* 2013;99:600-11.

9. Potet F, Egecioglu DE, Burridge PW, George AL Jr. GS-967 and Eleclazine Block Sodium Channels in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *Mol Pharmacol*. 2020;98:540-547.

10. Gima K, Rudy Y. Ionic current basis of electrocardiographic waveforms: a model study. *Circ Res*. 2002;90:889-96.

Figure legends

Figure 1. Pluripotency and SCN5A variant validation in hiPSCs, and characterization of derived cardiomyocytes.

A. Transcript expression of pluripotency markers: NANOG and OCT3/4, in newly described hiPSCs as compared to fibroblasts (Fibro).

B. Representative immunostainings of TRA1-60 (red) and OCT4 (green) in hiPSCs.

C. Percentage of hiPSCs expressing SSEA4 and TRA1-60 evaluated by flow cytometry.

D. Genomic sequence chromatograms validating (right) the 5164A>G *SCN5A* variant carried by BrS1⁺ and (left) the *SCN5A* 1983-1993 duplication carried by BrS2⁺ and Non-BrS, in the corresponding hiPSCs. E. Principal component analysis (PCA) of 39 hiPSC samples and their corresponding differentiated hiPSC-CMs, based on their expression pattern of 27106 analyzed transcripts (3'SRP data). All clones of each hiPSC lines are highlighted.

F. Correlation matrix of hiPSCs and hiPSC-CMs expression profiles. Yellow and orange indicate high and low correlation, respectively. Samples were clustered using an ascending hierarchical method with Pearson as metric and ward.D2 linkage.

G. Heatmap showing expression levels of 9661 differentially expressed genes between hiPSCs and hiPSC-CMs (same samples as in A). Genes were clustered using a hierarchical ascending method with an uncentered correlation metric and complete linkage. Yellow and blue indicate high and low levels respectively.

H. Illustrative immunostainings of Troponin I (green) in hiPSC-CMs. Nuclei were stained with DAPI (blue).

I. Percentages of nodal-like, atrial-like, and ventricular-like cells classified based on the analysis of spontaneous action potential recordings.

Figure 2. Differential gene expression profiles and variations in I_{Na} and $I_{Ca,Lr}$ in BrS-hiPSC-CMs as compared to controls.

A. Heatmap showing hierarchical clustering of expression profiles of 133 differentially expressed genes obtained by 3'SRP in control (Ctrl) and BrS hiPSC-CMs at day 28 of differentiation. A total of 27% were upregulated whereas 73% genes were downregulated in BrS hiPSC-CMs. Yellow and blue represent high and low expression levels, respectively. All clones of each hiPSC line are highlighted.

B. Gene Set Enrichment Analysis (GSEA) of gene variations obtained by 3'SRP shows gene sets with statistically altered expression patterns.

C. MindMap describing the transmembrane transporter activity alterations.

D. Expression levels of differentially expressed genes identified using high-throughput TaqMan (TLDA) in BrS hiPSC-CMs (n=14), compared to control hiPSC-CMs (n=12), and in Non-BrS hiPSC-CMs (n=4) *vs*.

BrS hiPSC-CMs. p-values: *, ** and *** or a, b and c: p<0.05, p<0.01 and p<0.001 vs. Ctrl or BrS, respectively (*t*-test).

E. Representative immunoblots for Na_v1.5 and Transferrin receptor (TFRC) in hiPSC-CMs (left panel). Ratios of Na_v1.5 expression levels (right panel, Tukey plot, n=8). * p<0.05 *vs.* control (Mann-Whitney test). Na_v1.5 decrease in hiPSC-CMs from 3 subjects, BrS2⁺ and Non-BrS (both carrying a stop codon in *SCN5A*), as well as BrS5⁻, harboring *RRAD* variant was observed.

F. Representative superimposed I_{Na} densities (inset: voltage-clamp protocol). Reduction was detected in BrS2⁺, BrS5⁻, and Non-BrS, as well as in BrS1⁺ hiPSC-CMs carrying the N1722D-SCN5A rare variant.

G. Peak I_{Na} densities measured in control (Ctrl), BrS and the non-affected carrier of *SCN5A* mutation (Non-BrS) hiPSC-CMs determined at -20 mV (Tukey plot). *** p<0.001 *vs.* control (Mann-Whitney test). H. Mean peak I_{Na} densities (pA/pF) *vs.* membrane potential (V_m) recorded in hiPSC-CMs. ****, \$\$\$, #### and ^^^^ p<0.0001 *vs.* control for BrS1⁺, BrS2⁺, BrS5⁻ and Non-BrS, respectively (Two-way ANOVA with Bonferroni post-hoc test).

I. Representative immunoblots for Ca_v1.2, the main pore-forming subunit of the cardiac L-type calcium channel, and Transferrin receptor (TFRC) in hiPSC-CMs (left panel). Ratios of Ca_v1.2 expression levels (right panel, Tukey plot, n=8). A decrease in Ca_v1.2 expression was solely observed in BrS5⁻ hiPSC-CMs, carrying a *RRAD*-variant. * p<0.05 *vs.* control (Mann-Whitney test).

J. Representative superimposed I_{Ca,L} densities (inset: voltage protocol).

K. Peak $I_{Ca,L}$ densities measured in control (Ctrl), BrS and the non-affected carrier of *SCN5A* mutation (Non-BrS) hiPSC-CMs determined at 0 mV (Tukey plot). A decrease in $I_{Ca,L}$ was observed in BrS2⁺, BrS4⁻ and, consistently with a previous description, in BrS5⁻.⁷ * p<0.05 and ** p<0.01 *vs.* control (Mann-Whitney test).

L. Mean peak $I_{Ca,L}$ densities (pA/pF) vs. membrane potential (V_m) recorded in hiPSC-CMs. *, # and \$ p<0.05, **, ## and \$\$ p<0.01 and ***, ### and \$\$\$ p<0.001 vs. control for BrS1⁺, BrS4⁻ and BrS5⁻, respectively (Two-way ANOVA with Bonferroni post-hoc test).

Figure 3. Increased early after depolarization (EAD) occurrence in all BrS ventricular-like hiPSC-CMs lines, linked to an increase in late sodium current.

A. Representative AP recordings, showing EADs in BrS lines only. Representative ventricular-like AP when paced at 700 ms cycle length and when artificial I_{K1} was injected (dynamic current-clamp). APs are defined as ventricular-like when (APD₃₀-APD₄₀)/(APD₇₀-APD₈₀)>1.45.

B. Percentage of ventricular-like hiPSC-CMs presenting at least 1 EAD, irrespective of the currentclamp conditions. * p<0.05, ** p<0.01 and *** p<0.001 vs. control (Fisher's exact test).

C. Representative $I_{Na,L}$ recordings from hiPSC-CMs, before (black) and after (grey) TTX application (inset: voltage protocol).

D. $I_{Na,L}$ (TTX-sensitive current) densities at -10 mV. * p<0.05 and *** p<0.001 vs. Ctrl (Mann-Whitney test), and ^{##} p<0.01 and ^{###} p<0.001 vs. Non-BrS (Mann-Whitney test).

E. Percentage of cells presenting $I_{Na,L}$ density greater than the 97th percentile value of $I_{Na,L}$ in the Ctrl hiPSC-CMs. ** p<0.01 and *** p<0.001 *vs*. control (Fisher's exact test). Indeed, an increase in $I_{Na,L}$ density was defined by values higher than the 97th percentile of the Ctrl hiPSC-CMs.

F. Representative example of $I_{Na,L}$ current recorded in BrS hiPSC-CMs before (black) and after (red) application with GS-458967 (300 nM), a specific $I_{Na,L}$ inhibitor (inset: voltage protocol).

G. Representative AP recordings from control and BrS hiPSC-CMs obtained before and after GS-458967 application.

H. Percentage of cells with EADs before and after GS-458967 application.

 Poincaré plots showing APD₉₀ of each AP (n+1) vs. APD₉₀ of its preceding one, before and after GS-458967 application.

Figure 4. Applying depolarizing ion current alterations from BrS hiPSC-CMs on an electrogram model to mimic BrS patient's ECG features.

A. Top: The right-ventricle electrogram model simulates the global electrical activity of a transmural wedge comprising 60 subendocardial, 45 midmyocardial, and 60 subepicardial human ventricular cells. Bottom: Representative electrogram showing the Q-like, R-like, S-like and T-like waves.

B. Ventricular transmural electrogram mathematical model of Ctrl (black), BrS2⁺ (pink) and Non-BrS (blue) illustrated based on hiPSC-CMs data of the relative variation in ion currents (I_{Na} , $I_{Ca,L}$ and $I_{Na,L}$) mean amplitude as compared to Ctrl lines. In accordance with patient's ECGs, applying BrS2⁺ ionic current changes prolonged the QRS-like complex, elevated and widened the ST-like segment, and inversed the T-like wave; and applying I_{Na} change identified in Non-BrS hiPSC-CMs only prolonged the QRS-like complex, similar to Non-BrS PCCD ECG.

C. Each currents I_{Na} , $I_{Ca,L}$ and $I_{Na,L}$ (from left to right) were sequentially corrected and the resulting electrograms are illustrated in green. Ctrl and BrS2⁺ electrograms are presented in black and pink respectively.

A consistent arrhythmogenic trait in Brugada syndrome cellular phenotype

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SUPPLEMENTAL MATERIAL

Supplemental methods:

Ethical Statement

The study was conducted according to the principles set forth under the Declaration of Helsinki (1989) and European guidelines for clinical and genetic research. Institutional review board approvals of the study were obtained before the initiation of patient enrollment. The study protocol was reviewed and approved by the regional *"comité de protection des personnes"* ethical committee (approval number: 2010-A01358-31). Regarding the patient-derived biological samples, signed informed consent allowing the experiments to be conducted has been received from all individuals. Any related health information was collected in compliance with applicable law/regulation and with any applicable policy of the ethics committee with jurisdiction over the biological sample collection. All biological samples and their related health information have been provided in coded form such that subjects cannot be identified directly. The provisions of French law, article L1110-4 of the *Code de la santé publique*, related to the privacy and confidentiality of information regarding patients, have been observed.

Study Population and design

BrS patients were enrolled according to the presence of a BrS ECG pattern (see below) and with a familial history of sudden death or syncope. Diagnosis of BrS was based on criteria from 2013¹ with the presence of a type-1 BrS ECG pattern, either spontaneous or induced by intravenous injection of class I antiarrhythmic drugs, in at least one right precordial lead (V1 or V2) positioned in the 2nd, 3rd or 4th intercostal space. Type-1 BrS ECG pattern was defined as a J point elevation higher than 0.2 mV, followed by a coved type ST segment elevation and ended with a negative T wave.

ECG analysis

Two physicians blinded to the clinical and genetic status reviewed all baseline ECGs. P wave, PQ interval, QRS, QT peak, QTend, QTc duration (corrected by Bazett formula), and Tpeak-Tend interval (TPE, time interval between the peak and the end of the T wave) were measured in D2, V1, V2 and V3. S-wave duration and amplitude were additionally measured in D1. All measurements were performed using Image J software as previously described.^{2,3} TPE in precordial lead, fragmented QRS, early-repolarization pattern and prominent R wave in lead aVR were determined as previously described.^{4,5} *Genetic analysis*

Patients were screened for mutations in genes previously described in cardiomyopathies and inherited arrhythmias.^{6,7} For this, genomic DNA of probands was extracted from peripheral blood lymphocytes by standard protocols. The DNA yields were assessed by measurements using Quant-IT[™] dsDNA Assay Kit, Broad Range (Life Technologies, Q33130). The purity of the DNA was assessed by

spectrophotometry (OD 260:280 and 260:230 ratios) using a Nanodrop instrument (Thermo Scientific). DNA integrity was assessed by separation in E-Gel[®] 96 Agarose Gels, 1% (Life Technologies, G700801). For multiplex amplification, we used the HaloPlex[™] Target Enrichment System (Agilent Technologies, 1-500 kb, ILMFST, 96 reactions, G9901B, Protocol Version D.2 (November, 2012)). A custom HaloPlex[™] design was used enabling high-throughput sequencing of the coding regions (exons ± 10 bp) of 20 genes previously associated with the BrS:^{6,7}

SCN5A	CACNA1C	RANGRF	KCNE1L
GPD1L	CACNB2	РКР2	KCNJ8
SCN1B	CACNA2D1	FGF12	ABCC9
SCN2B	KCNE3	SCN10A	KCNH2
SCN3B	KCND3	TRPM4	HCN4

Target enrichment and sequencing were performed as previously described.⁷ First, 200 ng of gDNA sample were digested in eight different restriction reactions, each containing two restriction enzymes, to create a library of gDNA restriction fragments. These gDNA restriction fragments were hybridized to the HaloPlex probe capture library. Probes were designed to hybridize and circularize targeted DNA fragments. During the hybridization process, Illumina sequencing motifs including index sequences were incorporated into the targeted fragments.

The circularized target DNA biotinylated HaloPlex probe complexes were captured on magnetic streptavidin beads. We proceeded to a ligation reaction of the circularized complexes followed by an elution reaction before PCR amplification. The amplified target DNA was purified using AMPure XP bead (Beckman Coulter, A63881). To validate enrichment of target DNA in each library sample by microfluidics analysis, we used the 2200 TapeStation (Agilent Technologies, G2964AA), with D1K ScreenTape (Agilent Technologies, 5067-5361), and D1K Reagents (Agilent Technologies, 5067-5362). We ensured that the majority of amplicons range from 175 to 625 bp. Finally, we quantified the library together with other libraries by qPCR using the KAPA Library Quantification Kit (Clinisciences, KK4854). Libraries were pooled in an equimolar concentration and DNA was then denatured with NaOH. Finally, the library pool was diluted to a final concentration of 9 pM before proceeding to 100-bp paired-end Illumina sequencing on HiSeq1500.

Raw sequence reads were aligned to the human reference genome (GRCh37) using BWA-MEM (version 0.7.5a) after removing sequences corresponding to Illumina adapters with Cutadapt v1.2. GATK was used for insertions and deletions (indel) realignment and base recalibration, following GATK DNAseq Best Practices. Variants were called for each sample separately using the Genome Analysis Toolkit GATK (UnifiedGenotyper version 2.8) Variants were considered as rare if the frequency was < 0.01% compared with the Gnomad database.⁸

2

Variants were considered as having a potential functional consequence if they were annotated with one or more of the following SO terms for at least one RefSeq transcript: "transcript ablation" (SO:0001893), "splice_donor_variant" (SO:0001575), "splice_acceptor_variant" (SO:0001574), "stop_gained" (SO:0001587), "frameshift_variant" (SO:0001589), "stop_lost" (SO:0001578), "initiator_codon_variant" (SO:0001582), "inframe_insertion" (SO:0001821), "inframe_deletion" (SO:0001822), "missense_variant" (SO:0001583), "transcript_amplification" (SO:0001889). Loss-offunction variants (nonsense variants, frameshift variants and splice site variants) were defined by the following SO terms: "stop gained", "frameshift variant", "splice donor variant", or "splice_acceptor_variant". The potential pathogenicity of variants was determined following the American College of Medical Genetics and Genomics (ACMG) guidelines using the CardioVAI tools from EnGenome.9

Generation and validation of hiPSCs

BrS5⁻ hiPSCs were previously used in a study revealing the role of a variant in *RRAD*, which encodes RAD GTPase, in BrS.¹⁰ BrS6⁻ hiPSC line, named iBrS1 in a previous study, was derived from a BrS patient with no defined causative variant.⁶ Four different control hiPSC lines were used, including 2 studied previously.^{10,11} BrS4⁻ cutaneous fibroblasts were reprogrammed using Stemgent mRNA reprogramming kit and pluriton medium. hiPSCs derived from dermal cells harvested from BrS1⁺ and BrS3⁻ as well as blood cells obtained from BrS2⁺ and his unaffected family relative (Non-BrS) were reprogrammed using Sendai virus method (Cytotune reprogramming Kit, Life technologies). Up to three clones of each hiPSC line were selected per patient. HiPSCs were maintained on matrigel-coated plates (0.05 mg/ml, BD Biosciences) with StemMACSTM iPS-Brew XF medium (Miltenyi Biotec). Pluripotency of each hiPSC clone was validated by qRT-PCR, immunostaining, and flow cytometry to verify the expression of endogenous pluripotent factors.

Validation of SCN5A rare variants and genome integrity

To validate *SCN5A* rare variants carried by BrS1⁺, BrS2⁺ and Non-BrS, genomic DNA from corresponding hiPSC clones was extracted using NucleoSpin® Tissue kit (MACHEREYNAGEL). Variants were verified by sequencing. Single nucleotide polymorphism (SNP) analysis of all hiPSC clones compared to their parental skin fibroblast cells, was used to confirm genome integrity after reprogramming. DNA was extracted from somatic and hiPSC samples using the QIAGEN QiaAmp kit, according to the manufacturer's recommendations. The gDNA was quantified using a Nanodrop instrument. 200ng of gDNA were outsourced to Integragen Company (Evry, France) for karyotype analysis using HumanCore-24-v1 SNP arrays. This array contains over 300,000 probes distributed throughout the genome with a median coverage of one probe every 5700 bases. All genomic positions were based on Human Genome Build 37 (hg19). Analysis was performed with GenomeStudio software. Chromosome abnormalities were determined by visual inspection of logR ratios and B-allele frequencies (BAF) values and

comparing parental cells with hiPSC-derived samples. LogR ratio, the ratio between observed and expected probe intensity, is informative of copy number variation (CNV, i.e. deletions/duplications), whereas BAF is informative of heterozygosity. SNP data were used to compute CNV. In particular, this type of chips allows detecting loss of heterozygosity (LOH), an important concern for hiPSCs, which is not detectable with classical CGH arrays.

Differentiation of hiPSCs into cardiomyocytes

Cardiomyocytes were differentiated from hiPSCs using the established matrix sandwich method.¹¹ Briefly, when cells reached 90% confluence, an overlay of Growth Factor Reduced Matrigel (0.033 mg/ml, BD Corning) was added. Differentiation was initiated 24 h later by culturing the cells in RPMI1640 medium (Life Technologies) supplemented with B27 (without insulin, Life Technologies), 2 mM L-glutamine (Life Technologies), 1% NEAA (Life Technologies), 100 ng/mL Activin A (Miltenyi), and 10 ng/mL FGF2 for 24 hours. On the next day, the medium was replaced by RPMI1640 medium supplemented with B27 without insulin, 2 mM L-glutamine, 1% NEAA, 10 ng/mL BMP4 (Miltenyi), and 5 ng/mL FGF2 for 4 days. By day 5, cells were cultured in RPMI1640 medium supplemented with B27 complete (Life Technologies), 2 mM L-glutamine and 1% NEAA and changed every two days. Presence of beating cells was considered as the primary hallmark of a successful cardiomyocyte differentiation.

Transcript expression analysis

Quantitative RT-PCR: Total RNA samples were isolated using the NucleoSpin RNA kit (MACHEREY-NAGEL). One μ g of tRNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. PCR amplification was performed using FAM labeled-TaqMan probes (Applied Biosystems) to verify the pluripotency of derived hiPSCs as compared to parental fibroblasts (OCT3/4 and NANOG). According to the $\Delta\Delta$ Ct method, all data were normalized to ACTB and represented relative to controls.

TaqMan Low Density array (TLDA): TLDA studies were conducted using beating clusters of hiPSC-CMs obtained from 12 samples of Ctrl, 4 samples of Non-BrS and 14 samples of BrS hiPSC-CMs. One μ g of RNA was reverse transcribed into cDNA using SuperScript IV Vilo Master Mix (Thermo Fisher Scientific). TLDA probe selection covered gene families implicated in cardiac ion channel expression and regulation, and cardiomyocyte structure (Table S3). Genes with average Ct > 32 in all compared groups were considered undetectable and excluded from the analysis (SCN10A and ABCC8). Average Ct of remaining genes for each sample was used for data normalization.¹²⁻¹⁴

3' Sequencing RNA Profiling (3'SRP): 3'SRP protocol was performed according to Kilens et al.¹⁵ Briefly, the libraries were prepared from 10 ng of total RNA. RNA samples were extracted from 26 BrS and 13 control hiPSC samples (a duplicate for each clone obtained at different cell passages) as well as their corresponding differentiated hiPSC-CMs. The mRNA poly(A) tail was tagged with universal adapters, well-specific barcodes and unique molecular identifiers (UMIs) during template-switching reverse

transcriptase. Barcoded cDNAs from multiple samples were then pooled, amplified and tagmented using a transposon-fragmentation approach which enriches for 3'ends of cDNA. A library of 350-800 bp was run on an Illumina HiSeq 2500 using a HiSeq Rapid SBS Kit v2 (50 cycles; FC-402-4022) and a HiSeq Rapid PE Cluster Kit v2 (PE-402-4002). Read pairs used for analysis matched the following criteria: all sixteen bases of the first read had quality scores of at least 10 and the first six bases correspond exactly to a designed well-specific barcode. The second reads were aligned to RefSeq human mRNA sequences (hg19) using bwa version 0.7.17. Reads mapping to several transcripts of different genes or containing more than 3 mismatches with the reference sequences were filtered out from the analysis. Digital gene expression profiles were generated by counting the number of UMIs associated with each RefSeq genes, for each sample. R package DESeq2 (Bioconductor) was used to normalize gene expression, and detect differentially expressed genes. Sample correlation matrix was performed using R package ComplexHeatmap (Bioconductor). Cluster 3.0 software was used to perform the differentially expressed gene heatmap. Gene Set Enrichment Analysis (GSEA) was performed using GeneTrail2. Statistically significant categories within the GO Molecular Process were identified using Kolomogorov-Smirnov test and p values were adjusted using the Benjamini and Hochberg method.

Protein expression analysis

Beating clusters of hiPSC-CMs were lysed in buffer composed of 1% TritonX-100, 100 mM NaCl, 50 mM Tris-HCl, 1 mM EGTA, 1 mM Na3VO4, 50 mM NaF, 1 mM PMSF and protease inhibitors cocktail (P8340, Sigma-Aldrish). Protein quantification was then conducted using PierceTM BCA Protein Assay Kit (Thermo Fisher). The lysates were denatured for 5 min at 65°C in a mixture of NuPAGE® Sample Reducing Agent (10X) and NuPAGE® LDS Sample Buffer (4X). 40 µg of each sample were loaded onto 12% precast polyacrylamide gels (Bio-Rad). After migration, the proteins were transferred onto Trans-Blot® TurboTM Nitrocellulose Transfer Packs (Bio-Rad). Membranes were saturated with 5% non-fat milk, then incubated with primary antibody (Anti-Na_v1.5: 14421S Cell Signaling; Anti-Cav1.2: AB5156 Sigma-Aldrich; Anti-TFRC:13-6890 Thermofisher) overnight followed by another incubation for 1 h with an adequate Horseradish peroxidase (HRP)-conjugated secondary antibody. Protein bands were detected using ECL detection system (Bio-Rad) and quantified using Image Lab software.

Immunostainings

To validate hiPSC pluripotency, hiPSC-CM differentiation into cardiac lineage as well as COS-7 cell expression, cells were dissociated and seeded onto 8-wells ibidi plates (Biovalley) coated with Matrigel (Corning). After 12 days following hiPSC-CM dissociation, and 24 h following COS-7 transfection, cultured cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X100 and blocked with 1% BSA. Immunofluorescent stainings were performed using appropriate primary antibodies (Anti-TRA1-60: 14-8863-80 eBioscience[™]; Anti-OCT4: 14-5841-80 eBioscience[™]; Anti-troponin I: sc-

15368 Santa Cruz; Anti-Na_v1.5: 14421S Cell Signaling) and their corresponding Alexa conjugated antibodies (Molecular Probes). DAPI was used for nuclear staining. Immunostainings were examined using an inverted epifluorescent microscope (Zeiss Axiovert 200M).

p.N1722D Nav1.5 study in COS-7 cells

The point mutation p.N1722D (c.1859G>A) in *SCN5A* was introduced using mutated oligonucleotide extension (QuikChange II XL Site-Directed Mutagenesis Kit) in *SCN5A* isoform 2 cDNA (GenBank Acc. Nb. NM_000335). The resulting plasmid was verified by complete sequencing of the cDNA insert. African green monkey kidney-derived cells COS-7, were transiently transfected with 0.4 μ g *SCN5A*-expressing plasmid (wild type or mutant) together with 0.4 μ g of Nav β 1 subunit (SCN1B) plasmid for a 35-mm Petri dish. Transfections were performed using 4 μ L JetPEI reagent (Polyplus Transfections, France) according to the manufacturer's instructions. Enhanced green fluorescent protein (eGFP)-encoding plasmid (1.2 μ g) was included to identify transfected cells. One day after transfection, cells were re-plated onto 35-mm Petri dishes for patch clamp experiments.

Electrophysiological assessment

Sodium current (I_{Na}) recordings in transfected COS-7 cells. Currents were recorded 2 days after transfection. Cells were superfused with a solution containing the following (in mM): 145 NaCl, 4 CsCl, 1 CaCl2, 1 MgCl2, 5 HEPES, and 5 glucose, pH=7.4 with NaOH. Patch pipettes were fabricated from borosilicate glass capillaries and had resistances between 1.5 and 2 MΩ when filled with pipette solution (in mM): 90 KCl, 45 K-Gluconate, 10 NaCl and 10 HEPES, pH=7.2 with CsOH. All recordings were made at room temperature (20°C-22°C), after capacitance and series resistance compensation, using an Axopatch 200B amplifier controlled by Axon pClamp 10.6 software through an A/D converter (Digidata 1440A). Data were analyzed using Clampfit 10.6 software (all Molecular Devices).

Current- and voltage-clamp in hiPSC-CMs. Cardiomyocytes were dispersed as single cells around day 20 of differentiation, for 20 min in collagenase II (200 U/mL; Gibco) at 37°C. Ten to twelve days after dissociation, spontaneously beating cells were used for patch-clamp recordings. All experiments were conducted at 37°C. Data were collected from a minimum of 3 independent differentiations.

Action potential (AP) recordings. Using amphotericin-B perforated-patch configuration, APs were acquired with the same amplifier and converter as above in hiPSC-CMs cells bathed in a Tyrode solution containing (in mM): 140 NaCl, 4 KCl, 1 CaCl2, 0.5 MgCl2, 10 glucose, 10 HEPES; pH 7.4 (NaOH). Borosilicate glass pipettes (2-3 M Ω of tip resistance) were filled with a solution containing (in mM): 125 K-Gluconate, 20 KCl, 5 NaCl, 5 HEPES; pH 7.2 (KOH) and 0.22 amphotericin-B. We first recorded spontaneous APs in order to determine, for each individual included in the study, the proportion of nodal-like, atrial-like and ventricular-like, as previously described.¹¹ Then to overcome limited I_{K1} contribution during hiPSC-CM AP, artificial I_{K1} was injected using dynamic patch-clamp.¹⁶ Both cell stimulation and I_{K1} injection were realized using a custom-made software running on RT-Linux and an

A/D converter (National Instrument PCI-6221) connected to the current command of the amplifier. The AP parameters measured were the maximum diastolic potential (MDP), the maximum upstroke velocity of phase 0 depolarization (dV/dt_{max}), the AP amplitude and the AP duration at different levels of full repolarization, from 30% (APD30) to 90% (APD90), as previously described.¹¹ Following I_{K1} injection, at a peak outward density that sets the membrane potential between -80 to -85mV for all cells, action potential classification into ventricular type was assessed by (APD30-APD40)/(APD70-APD80)>1.45 reflecting the presence of a plateau phase. Cells were paced with a 1-ms 30-35 pA/pF stimulation pulse at 700 ms of cycle length. BrS being a ventricular arrhythmic disease, the analyses were focused on ventricular-like AP. Data from 7 consecutive APs were averaged. GS-458967 (Gilead Sciences) specifically inhibiting I_{Na,L}, was solubilized in DMSO and used at 300 nM during AP recording in a solution containing (in mM): 140 NaCl, 4 KCl, 1 CaCl2, 0.5 MgCl2, 30 mannitol, 10 HEPES; pH 7.4 (NaOH).

Current recordings. I_{Na} and I_{Ca,L} measurements were recorded in the ruptured-patch configuration and low-pass filtered at, respectively, 10 KHz and 3 KHz using a VE-2 amplifier (Alembic Instrument, Qc, Canada). Cells were bathed using a Tyrode solution containing (in mM): 130 NaCl, 10 CsCl, 1.8 CaCl2, 1.2 MgCl2, 11 glucose and 5 HEPES; pH 7.4 (NaOH). Holding potentials were set to -80 mV and -100 mV, respectively. The series resistance was compensated. Current densities and gating properties were measured using appropriate voltage protocols shown in the relevant figures. After leak subtraction, current densities were calculated by dividing current amplitude by membrane capacitance. Voltage-dependence of activation and inactivation curves were fitted with a Boltzmann function (y=[1+exp{-(V-V1/2)/K}]-1), where V1/2 is the half-maximal voltage of (in)activation and K is the slope factor.

For transient **sodium current (I_{Na})** recording, a local gravity microperfusion system allowed application of an extracellular solution containing (in mM): 20 NaCl, 110 CsCl, 1.8 CoCl2, 1.2 MgCl2, 30 mannitol and 5.0 HEPES; pH 7.4 (CsOH). The pipette solution contained (in mM): 3 NaCl, 133 CsCl, 2 MgCl2, 2 Na2ATP, 2 TEACl, 10 EGTA, 5 HEPES; pH 7.2 (CsOH).

Late sodium current $(I_{Na,L})$ was measured as a TTX-sensitive current (Tetrodotoxin Citrate, TOCRIS Bioscience) using an ascending voltage-ramp protocol. The same pipette solution as for peak I_{Na} recording was used. The extracellular solution used in the local gravity microperfusion system had the following composition (in mM): 130 NaCl, 10 CsCl, 1.8 CoCl2, 1 MgCl2, 30 mannitol, 10 HEPES; pH 7.4 (CsOH). TTX was used at a concentration of 0.03 mM.

Calcium current (I_{Ca,L}) was recorded from cells perfused an extracellular solution containing (in mM): 160 TEACI, 5 CaCl2, 1 MgCl2, 1 MgCl2, 20 mannitol, 10 HEPES, and 0.01 TTX; pH 7.4 (CsOH). The pipette solution contained (in mM): 5 NaCl, 145 CsCl, 2 CaCl2, 5 EGTA, 5 MgATP, 10 HEPES; pH 7.2 (CsOH).

Mathematical electrogram modeling

Right-ventricle electrogram was calculated as in Gima and Rudy,¹⁷ by simulating a heterogeneous transmural wedge (right ventricular outflow tract, 1 Hz, 500th beat shown). This model aims at mimicking the global electrical activity of a row of 165 subendocardial, midmyocardial and subepicardial human ventricular cells.^{18,19} In the original model used by Gima and Rudy, a slow inactivation gate (j) of the Na+ channel was added to include the property of slow recovery,²⁰ without modifying fast inactivation (realized with h gate). A few modifications were operated. Since this 'j' inactivation was originally as fast as 'h' inactivation, it prevents the appearance of a persistent current when altering the 'h' gate. So we slowed down this 'j' inactivation, but left the kinetics of recovery intact, by modifying the following equation:

If $V \ge -50$ beta_j=0.035*exp(+0.008*p->v)/(1.0+exp(-0.048*(p->v+77.329)).

Thus, in the modified model, any incomplete fast inactivation (for instance, h parameter varies between 1 and 0.166 for BrS2⁺, instead of 1 and zero for Ctrl) gives rise to a late Na⁺ current similar to that observed in Figure 3C (see also Figure below) resulting in the following equation.



Impact of incomplete inactivation on late sodium current in a simulated Ramp protocol. Ctrl: h parameter varies between 1 and 0.1^7 BrS: h varies between 1 and 0.116. The ratio of maximal I_{Na,L} current in BrS/Ctrl is 5.4, as in Figure 3C for BrS2⁺.

Noteworthy, this modification does not change inactivation properties of the Na⁺ current in the WT model because fast inactivation (h) is not modified.

Since only a subset of hiPSC-CMs present the late Na⁺ current, we performed simulations with the late Na⁺ current only in midmyocardial cells, giving rise to a J point elevation followed by coved ST segment.¹⁸

Since decreasing I_{Na} by as much as 73-75% (BrS2⁺ and Non-BrS) was preventing conduction in the Gima and Rudy model, a slighter 30% decrease in I_{Na} was applied.

Statistical analysis

Results are expressed as mean \pm SEM. Comparisons were made by use of Mann-Whitney test, Student *t*-test, or two-way ANOVA with Bonferroni post-hoc test for repeated measures. Correlations were investigated based on correlation coefficient r_s of Spearman.

Values of p < 0.05 were considered statistically significant. Statistical analyses were performed with GraphPad Prism software.

Supplemental results:

Patient characteristics

Six patients affected by type I BrS (BrS1-6) with a familial history of SCD or syncope were selected, among which two carrying *SCN5A* variants (marked with a ⁺ symbol in BrS1⁺ and BrS2⁺).

Patient #1 (BrS1⁺) was a 35-year-old male with a history of recurrent near-syncope. He presented with a spontaneous BrS ECG pattern. After induction of ventricular fibrillations (VF) during electrophysiological study, an implantable cardioverter-defibrillator (ICD) was implanted with no recurrence of syncope or VF during a 14-year follow-up. Five of his relatives also exhibited a BrS ECG pattern.

Patient #2 (BrS2⁺) was an asymptomatic 55-year-old male whose brother died suddenly at age 38. He presented a spontaneous BrS ECG pattern with occurrence of VF during an electrophysiological study. An ICD was implanted with no occurrence of syncope or VF during a 13-year follow-up. Six of his relatives presented with a BrS ECG pattern including his son who exhibited one spontaneous episode of VF.

Patient #3 (BrS3⁻) was a 35-year-old male who suffered from syncope at night and presented with a spontaneous BrS ECG pattern with VF occurrence during an electrophysiological study. Despite hydroquinidine administration, several episodes of VF were reported. Endocardial catheter ablation of premature ventricular beats was performed without further recurrences under hydroquinidine therapy. Two of his relatives presented with a BrS ECG pattern.

Patient #4 (BrS4⁻) was a 44-year-old male who presented episodes of unexplained syncope at rest. Ajmaline test revealed a BrS ECG pattern. Electrophysiological study did not induce any arrhythmia. Due to familial history of SCD and recurrent syncope, an ICD was implanted without recurrence of syncope or VF during a 13-year follow-up. Type-1 BrS ECG was identified in 8 additional family members.

Patient #5 (BrS5⁻) was a 41-year-old male, previously described by Belbachir *et al*,¹⁰ who presented recurrent near-syncopes with palpitations and spontaneous BrS ECG pattern. Electrophysiological study induced VF. An ICD was implanted with no recurrence of syncope or VF during a 16-year follow-up. Familial screening identified 6 relatives with a BrS ECG pattern and one with unexplained SCD at age 41.

Patient #6 (BrS6⁻) was a 42-year-old male, previously described by Veerman *et al.*⁶ While he had a spontaneous BrS ECG pattern, he presented an unexplained syncope, and suffered from an outof-hospital cardiac arrest at night. Three of his relatives presented with a BrS phenotype after ajmaline administration.

An additional Patient #7, not affected by Brugada syndrome (Non-BrS), was recruited. He was a 67-year-old male diagnosed with progressive cardiac conduction defect (PCCD). He was the nephew of patient BrS2⁺. His ECG displayed broad PR interval and prolonged QRS duration, however, no ST segment elevation was detected even after challenge with flecainide.

Four control subjects were also included. One subject who was a relative of BrS5⁻ patient,¹⁰ in whom BrS was excluded after sodium channel blocker challenge and three other unrelated healthy extra familial controls, including one previously described¹¹ and 2 others with different ethnicity.²¹

Representative patient ECGs are presented in Figure S1. Description of patients and their corresponding ECG measurements are depicted in Table S1 and Table S2 respectively.

Genetic characterization

Genetic screening of the coding regions of 20 genes, selected according to the American College of Medical Genetics and Genomics (ACMG) guidelines, revealed *SCN5A* pathogenic variants in only two of the six BrS patients. BrS1⁺ carried a c.5164A>G missense rare variant, resulting in asparagine to aspartic acid substitution at position 1722 in the extracellular connecting loop, between segment 5 and 6 in domain IV forming the pore region of Na_v1.5 (p.N1722D). BrS2⁺ and his nephew (Non-BrS) affected by PCCD carried a 10bp duplication (c.1983–1993dup) in *SCN5A* creating a stop codon (p.A665G-fsX16). Both these variants had not been functionally investigated yet. BrS5⁻ patient presented a previously described rare genetic variant in the *RRAD* gene (p.R211H)¹⁰ whereas no genetic variation was identified in BrS candidate genes in BrS3⁻, BrS4⁻ or BrS6⁻ patients (Table S1).

Generation and characterization of hiPSCs and hiPSC-CMs

Somatic cells were obtained from all studied subjects and were reprogrammed into corresponding hiPSC lines. BrS5⁻, BrS6⁻ and the control hiPSC lines have been previously characterized.^{6,10,11,21} For each of the other 5 newly generated hiPSC lines (BrS1 to 4 and Non-BrS), up to three independent clones were amplified and characterized. SNP analysis verified that control and mutated hiPSC lines were free from any genomic aberrations compared to parental somatic cells (data not shown). The expression of the pluripotent stem cell markers was verified (Figure 1A, 1B and 1C). Genetic screening confirmed that the lines arising from patients carrying *SCN5A* genetic variants, BrS1⁺, BrS2⁺ and Non-BrS harbored the corresponding heterozygous rare variant (Figure 1D).

hiPSC differentiation process into cardiomyocytes was also validated at the transcriptional level. 3'SRPbased global transcriptomic analysis of control and BrS hiPSCs and hiPSC-CMs showed that cells clustered based on their stage (hiPSCs on one side and hiPSC-CMs on the other side) and independently from their genetic background (Figure 1E). Correlation analysis also showed that samples correlated according to their stage, with all hiPSC samples being correlated to other hiPSC samples and similarly for hiPSC-CM samples (Figure 1F). Finally, both Ctrl and BrS hiPSC-CMs presented a comparable global change in gene expression as compared to hiPSC stage (Figure 1G). Immunostaining analysis showed

that striated troponin I, a cardiac and muscular specific cytoskeletal protein, was similarly present in all hiPSC-CM lines (Figure 1H). Spontaneous APs, in absence of I_{K1} injection, were classified based on MDP, dV/dt_{max}, AP duration and morphology into nodal-like, atrial-like and ventricular-like, as previously described.¹¹ The proportion of each cell type was similar between all hiPSC-CM lines, with the ventricular-like type forming the majority of explored hiPSC-CMs (Figure 1I). Altogether, these data confirmed that Ctrl, BrS, and Non-BrS hiPSCs differentiated similarly into cardiomyocytes, and therefore a comparative electrophysiological analysis could be performed to unveil a potential common cellular phenotypic trait of BrS hiPSC-CMs.

Supplemental references:

1. Priori SG, Wilde AA, Horie M, et al. HRS/EHRA/APHRS Expert Consensus Statement on the Diagnosis and Management of Patients with Inherited Primary Arrhythmia Syndromes. *Heart Rhythm*. 2013;10:1932-63.

2. Therasse D, Sacher F, Babuty D, et al. Value of the sodium-channel blocker challenge in Brugada syndrome. *Int J Cardiol*. 2017;245:178-80.

3. Therasse D, Probst V, Gourraud J-B. Sodium channel blocker challenge in Brugada syndrome: Role in risk stratification. *Int J Cardiol*. 2018;264:100-1.

4. Gourraud J-B, Barc J, Thollet A, et al. Brugada syndrome: Diagnosis, risk tratification and management. *Arch Cardiovasc Dis.* 2017;110:188-95.

5. Berthome P, Tixier R, Briand J, et al. Clinical presentation and follow-up of women affected by Brugada syndrome. *Heart Rhythm.* 2018;16:260-267.

6. Veerman CC, Mengarelli I, Guan K, Stauske M, Barc J, Tan HL, Wilde AAM, Verkerk AO, Bezzina CR. hiPSC-derived cardiomyocytes from Brugada Syndrome patients without identified mutations do not exhibit clear cellular electrophysiological abnormalities. *Sci Rep.* 2016;6:30967.

7. Scouarnec SL, Karakachoff M, Gourraud J-B, Lindenbaum P, Bonnaud 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, Haïssaguerre M, Kyndt F, Schmitt S, Bézieau S, Marec HL, Dina C, Schott J-J, 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;ddv036.

8. Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, Wang Q, Collins RL, Laricchia KM, Ganna A, Birnbaum DP, Gauthier LD, Brand H, Solomonson M, Watts NA, Rhodes D, Singer-Berk M, Seaby EG, Kosmicki JA, Walters RK, Tashman K, Farjoun Y, Banks E, Poterba T, Wang A, Seed C, Whiffin N, Chong JX, Samocha KE, Pierce-Hoffman E, Zappala Z, O'Donnell-Luria AH, Minikel EV, Weisburd B, Lek M, Ware JS, Vittal C, Armean IM, Bergelson L, Cibulskis K, Connolly KM, Covarrubias M, Donnelly S, Ferriera S, Gabriel S, Gentry J, Gupta N, Jeandet T, Kaplan D, Llanwarne C, Munshi R, Novod S, Petrillo N, Roazen D, Ruano-Rubio V, Saltzman A, Schleicher M, Soto J, Tibbetts K, Tolonen C, Wade G, Talkowski ME, Consortium TGAD, Neale BM, Daly MJ, MacArthur DG. Variation across 141,456 human exomes and genomes reveals the spectrum of loss-of-function intolerance across human protein-coding genes. *bioRxiv*. 2019;531210.

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, 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 Off J Am Coll Med Genet*. 2015;17:405-24.

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10. Belbachir N, Portero V, Al Sayed ZR, et al. RRAD mutation causes electrical and cytoskeletal defects in cardiomyocytes derived from a familial case of Brugada syndrome. *Eur Heart J.* 2019;ehz308.

11. Es-Salah-Lamoureux Z, Jouni M, Malak OA, et al. HIV-Tat induces a decrease in IKr and IKs via reduction in phosphatidylinositol-(4,5)-bisphosphate availability. *J Mol Cell Cardiol*. 2016;99:1-13.

12. Wang W-X, Danaher RJ, Miller CS, Berger JR, Nubia VG, Wilfred BS, Neltner JH, Norris CM, Nelson PT. Expression of miR-15/107 family microRNAs in human tissues and cultured rat brain cells. *Genomics Proteomics Bioinformatics*. 2014;12:19-30.

13. Mestdagh P, Van Vlierberghe P, De Weer A, Muth D, Westermann F, Speleman F, Vandesompele J. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol.* 2009;10:R64.

14. Bockmeyer CL, Säuberlich K, Wittig J, Eßer M, Roeder SS, Vester U, Hoyer PF, Agustian PA, Zeuschner P, Amann K, Daniel C, Becker JU. Comparison of different normalization strategies for the analysis of glomerular microRNAs in IgA nephropathy. *Sci Rep.* 2016;6:31992.

15. Kilens S, Meistermann D, Moreno D, et al. Parallel derivation of isogenic human primed and naive induced pluripotent stem cells. *Nat Commun.* 2018;9:360.

16. Meijer van Putten, Mengarelli I, Guan K, et al. Ion channelopathies in human induced pluripotent stem cell derived cardiomyocytes: a dynamic clamp study with virtual IK1. *Front Physiol*. 2015;6:7.

17. Gima K, Rudy Y. Ionic current basis of electrocardiographic waveforms: a model study. *Circ Res.* 2002;90:889-96.

18. Zygmunt AC, Eddlestone GT, Thomas GP, et al. Larger late sodium conductance in M cells contributes to electrical heterogeneity in canine ventricle. *Am J Physiol Heart Circ Physiol*. 2001;281:H689-697.

19. Li GR, Feng J, Yue L, Carrier M. Transmural heterogeneity of action potentials and Ito1 in myocytes isolated from the human right ventricle. *Am J Physiol*. 1998;275:H369-377.

20. Luo CH, Rudy Y. A model of the ventricular cardiac action potential. Depolarization, repolarization, and their interaction. *Circ Res.* 1991;68:1501-26.

21. Al Sayed ZR, Canac R, Cimarosti B, Bonnard C, Gourraud JB, Hamamy H, Kayserili H, Girardeau A, Jouni M, Jacob N, Gaignerie A, Chariau C, David L, Forest V, Marionneau C, Charpentier F, Loussouarn G, Lamirault G, Reversade B, Zibara K, Lemarchand P, Gaborit N. Human model of IRX5 mutations reveals key role for this transcription factor in ventricular conduction. *Cardiovasc Res*. 2020;8:cvaa259.

22. Morita H, Kusano KF, Miura D, et al. Fragmented QRS as a marker of conduction abnormality and a predictor of prognosis of Brugada syndrome. *Circulation*. 2008;118:1697-1704.



Figure S1. Electrocardiogram of all subjects from whom hiPSCs were derived

Leads V1 to V3 from electrocardiogram of Non-BrS and BrS1-6 patients. + and – signs refer to the presence and absence of a *SCN5A* alteration variant, respectively. Type 1 BrS characteristics are visible in all BrS ECG and conduction defect is visible in Non-BrS ECG (right branch block).





Figure S2: Electrophysiological characterization of N1722D-SCN5A-expressing COS-7 cells.

The effects of N1722D-*SCN5A* variant present in BrS1⁺ hiPSC-CMs were characterized using COS-7 cells transfected with plasmids expressing either wild type (WT-*SCN5A*) or N1722D-*SCN5A* cDNA.

A. Illustrative immunostainings of Na_v1.5 (green) in COS-7 cells transfected with WT *SCN5A* and N1722D-*SCN5A* cDNA, showing robust Na_v1.5 expression of the variant at the cell surface. Nuclei were stained with DAPI (blue). B. Representative whole-cell current recorded in COS-7 cells overexpressing wild type (WT) and N1722D-*SCN5A* cDNA (left panel; voltage protocol in inset). Mean peak I_{Na} current densities (pA/pF) vs. membrane potential (Vm) (right panel). ** p<0.01 vs. WT (Two-way ANOVA with Bonferroni post-hoc test). C. Peak I_{Na} current densities, measured at -20 mV (Tukey plot), unveiling a significant reduction, by about 2 folds, of I_{Na} in N1722D-*SCN5A* transfected-COS-7 cells. *** p<0.001 vs. control (*t*-test).

D. I_{Na} voltage-dependence of inactivation and activation. For inactivation, I_{Na} was normalized to maximum (I/Imax), and plotted as a function of the potential of conditioning pulse that preceded the test pulse to -20 mV (inactivation; inset: voltage protocol). For activation, GNa (i.e. $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na+ ions) was normalized to maximum, and plotted as a function of Vm, the potential of the test pulse I_{Na} voltage dependence of activation. GNa (as $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na+ ions) was normalized to maximum, and plotted as a function of Vm, the potential of the test pulse I_{Na} voltage dependence of activation. GNa (as $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na⁺ ions) was normalized to maximum, and plotted as a function of Vm, the potential of the test pulse (activation: same voltage protocol as in B). This analysis did not reveal any modification of steady-state activation and inactivation, as in BrS1⁺ hiPSC-CMs.





Figure S3. I_{Na} and $I_{Ca,L}$ steady-state activation and inactivation gating properties in BrS hiPSC-CMs as compared to controls.

A. Left: I_{Na} voltage-dependence of inactivation. I_{Na} was normalized to its maximum value, and plotted as a function of the potential of conditioning pulse that preceded the -20-mV test pulse (inset: voltage protocol).

Right: I_{Na} voltage-dependence of activation. GNa (as $I_{Na}/(Vm-E_{Na})$, E_{Na} being the equilibrium potential for Na⁺ ions) was normalized to its maximum value, and plotted as a function of the potential of the test pulse (Vm; inset: voltage protocol).

B. I_{Ca,L} voltage-dependence of activation and inactivation (inset: voltage protocol).

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Figure S4:

Figure S4. Ventricular action potential (AP) parameters in BrS hiPSC-CMs as compared to controls.

A. Representative ventricular-like AP when paced at 700 ms cycle length and when artificial I_{K1} was injected (dynamic current-clamp). APs are defined as ventricular-like when $(APD_{30}-APD_{40})/(APD_{70}-APD_{80})>1.45$.

B. Maximum upstroke velocity (dV/dt_{max}) of ventricular-like APs (Tukey plot). Conditions as in A. *p<0.05 *vs.* control (Mann-Whitney test).

C. AP overshoot from ventricular-like hiPSC-CMs. Conditions as in A. * p<0.05; vs control (t-test).

The AP maximum upstroke velocity (dV/dt_{max}) and overshoot were reduced in hiPSC-CMs presenting a reduction in I_{Na} .

D. Ventricular-like AP duration (APD) at 30%, 50% and 90% of full repolarization, showing that consistent with the absence of QT duration modification in BrS patients' ECGs, no difference in AP duration was observed between BrS and Ctrl hiPSC-CMs. Conditions as in A.

E. Beating frequencies of investigated cell lines. Box plots presenting peak-to-peak durations between action potentials, averaged for all spontaneously recorded action potentials (p = ns; One-way Anova test)





Figure S5. Proportions of ventricular action potentials with or without EADs.

Percentage of ventricular-like hiPSC-CMs presenting at least 1 EAD, irrespective of the current-clamp conditions, for each investigated clone of each hiPSC line.

Supplemental Tables:

Table S1. Patient description

Subject	Genetic variant	Clinical Phenotype
Ctrl1	E1	-
Ctrl2	-	-
Ctrl3	-	-
Ctrl4	-	
BrS1 ⁺	SCN5A (p.N1722D)	BrS
BrS2 ⁺	SCN5A (p.A665G-fsX16)	BrS
BrS3 ⁻	-	BrS
BrS4 ⁻	-	BrS
BrS5	RRAD (p.R211H)	BrS
BrS6 ⁻	-	BrS
Non-BrS	SCN5A (p.A665G-fsX16)	PCCD

Table S2. ECG patient characteristics.

ECGs were performed in leads D1, D2, V1, V2, V3 and recordings used the following parameters: 25 mm/s, 0.1 mV/mm.

RR interval duration (RR); S wave duration and amplitude (S); P wave duration (P); PR interval duration (PR); QRS duration (QRS); QT peak interval (QTp); QT end interval (QTe); Tpeak-to -Tend interval (TPE); J wave amplitude (J); Early Repolarization Pattern (ERP); Fragmented QRS according to Morita H et al.²² (Frag); Concave aspect of the ST segment elevation (Concave aspect); Yes (y); No (n).

		D1			D2				V1		V2		V3		
	RR ms	S ms	S mm	Pms	PR ms	QRS ms	QTp ms	QTe ms	TPE ms	Jmm	TPE ms	J mm	TPE ms	Jmm	TPE ms
Ctrl	837.8	40.5	2.4	101.4	168.9	81.1	290.5	351.4	60.8	0.3		0.7	81.1	0.7	81.1
BrS1+	709.5	47.3	2.2	128.4	229.7	121.6	297.3	371.6	74.3	4.6	60.8	4.6	74.3	0.5	47.3
BrS2+	959.7	67.1	5.7	114.1	221.5	107.4	295.3	375.8	80.5	3.0	100.7	4.2	107.4	1.8	100.7
BrS3-	797.3			108.1	189.2	121.6	277.0	378.4	101.4	2.2	108.1	1.7	195.9	0.5	121.6
BrS4-	798.7	40.3	1.2	94.0	174.5	94.0	275.2	349.0	73.8	b.8	60.4	2.9	47.0	2.3	80.5
BrS5-	831.1	27.0	1.0	94.6	162.2	81.1	297.3	364.9	67.6	1.9	54.1	4.7	101.4	2.4	128.4
BrS6-	763.2	96.5	3.1	105.3	166.7	131.6	289.5	377.2	87.7	3.1	122.8	1920	149.1	-0.9	105.3
Non-BrS	771.8	13.4	0.7	87.2	187.9	100.7	255.0	328.9	73.8	0.3	67.1	1.0	60.4	1.8	80.5

	ERP	Frag	N	egative T wa	ve	0	concave aspec	ct
	y/n		V1	V2	V3	V1	V2	V3
Ctrl	n	n	n	n	n	n	n	n
BrS1+	n	n	Y	У	n	У	У	n
BrS2+	n	n	Y	У	n	У	У	n
BrS3-	У	n	Y	У	n	У	n	n
BrS4-	n	n	Y	n	n	n	n	n
BrS5-	n	n	Y	У	У	У	У	n
BrS6-	n	n	Y	У	n	n	n	n
Non-BrS	n	n	Y	n	n	n	n	n

6	ene symbol	protein symbol	Gene name	Assav ID	Category	
7	BCCB	SLIP1	ATP-binding cassette sub-family C (CETR/MPP) member 8	Hc01093761 m1	Potassium (K ⁺) channel	
Ľ	RCCO	5001	ATP binding cassette, sub-family C (CETP/MRP), member 0	Hc00345832 m1	Potassium (K ⁺) channel	
Ľ	10003	No/K ATRaco a2	ATPace Na /// transporting alpha 2 polymontide	H:000E8036 m1		
Ľ	70101	Na/K-ATPase as	ATPase, Na+/K+ transporting, apria 5 polypeptide	1500558050_111	Na ⁺ /K ⁺ ATDasa	
Ľ	TP1B1	Na/K-ATPase D1	A i Pase, Na+/K+ transporting, beta 1 polypeptide	HSUU426868_g1	Na /K AlPase	
Ľ		SERCAZ	A Pase, Ca++ transporting, cardiac muscle, slow twitch 2	HS00544877_m1	Calcium (Ca) regulator	
Ľ	TP2A3	SERCA3	ATPase, Ca++ transporting, ubiquitous	Hs00193090_m1	Calcium (Ca ⁺⁺) regulator	
ŕ	TP2B4	Ca ATPase4	ATPase, Ca++ transporting, plasma membrane 4	Hs00608066_m1	Calcium (Ca'') regulator	
	ACNA1C	Cav1.2	calcium channel, voltage-dependent, L type, alpha 1C subunit	Hs00167681_m1	Calcium (Ca++) channel	
C	ACNA1D	Cav1.3	calcium channel, voltage-dependent, L type, alpha 1D subunit	Hs00167753_m1	Calcium (Ca++) channel	
C	ACNA1G	Cav3.1	calcium channel, voltage-dependent, alpha 1G subunit	Hs00367969_m1	Calcium (Ca++) channel	
C	ACNA1H	Cav3.2	calcium channel, voltage-dependent, alpha 1H subunit	Hs01103527_m1	Calcium (Ca++) channel	
c	ACNA2D1	Cava2d1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	Hs00984856_m1	Calcium (Ca++) channel	
c	ACNA2D2	Cava2d2	calcium channel, voltage-dependent, alpha 2/delta subunit 2	Hs01021049_m1	Calcium (Ca++) channel	
c	ACNB2	Cavb2	calcium channel, voltage-dependent, beta 2 subunit	Hs01100744_m1	Calcium (Ca++) channel	
c	ALM1	CALM1	calmodulin 1 (phosphorylase kinase, delta)	Hs00300085_s1	Calcium (Ca ⁺⁺) regulator	
c	ALM3	CALM3	calmodulin 3 (phosphorylase kinase, delta)	Hs00968732_g1	Calcium (Ca ⁺⁺) regulator	
	ASQ2	CASQ2	calsequestrin 2 (cardiac muscle)	Hs00154286 m1	Calcium (Ca ⁺⁺) regulator	
	JA1	Cx43	gap junction protein, alpha 1, 43kDa (connexin 43)	Hs00748445 s1	Connexin	
6	JA5	Cx40	eap junction protein, alpha 5, 40kDa (connexin 40)	Hs00270952 m1	Connexin	
a	JA7	Cx45	gap junction protein, alpha 7, 45kDa (connexin 45)	Hs00271416_s1	Connexin	
	102	Cv30.2	gan junction Protein delta 3 31 9kDa	Hc00987388 c1	Connexin	
Ľ.		UCN1	bunger participation activated cyclic puckettide gated petaccium channel 1	Hs01085412 m1	Cation channel	
Ľ			hyperpolarization activated cyclic nucleotide-gated potassium channel 1	11501005412_111	Cation channel	
Ľ		HCN2	hyperpolarization activated cyclic nucleotide-gated polassium channel 2	HS00606903_1111	Cation channel	
Ľ	ICN3	HCN3	nyperpolarization activated cyclic nucleotide-gated potassium channel 3	HS00380018_m1	Cation channel	
ľ	ICN4	HCN4	nyperpolarization activated cyclic nucleotide-gated potassium channel 4	Hs00975492_m1	Cation channel	
1	IPR1	ITPR1	inositol 1,4,5-triphosphate receptor, type 1	Hs00181881_m1	Calcium (Ca ⁺⁺) regulator	
ľ	TPR3	ITPR3	inositol 1,4,5-triphosphate receptor, type 3	Hs00609908_m1	Calcium (Ca ') regulator	
P	CNA2	Kv1.2	potassium voltage-gated channel, shaker-related subfamily, member 2	Hs00270656_s1	Potassium (K ⁺) channel	
P	CNA4	Kv1.4	potassium voltage-gated channel, shaker-related subfamily, member 4	Hs00937357_s1	Potassium (K ⁺) channel	
P	CNA5	Kv1.5	potassium voltage-gated channel, shaker-related subfamily, member 5	Hs00969279_s1	Potassium (K ⁺) channel	
Þ	CNA7	Kv1.7	potassium voltage-gated channel, shaker-related subfamily, member 7	Hs00361015_m1	Potassium (K ⁺) channel	
þ	CNAB2	Kvb2	potassium voltage-gated channel, shaker-related subfamily, beta member 2	Hs00186308_m1	Potassium (K⁺) channel	
Þ	CNAB3	Kvb3	potassium voltage-gated channel, shaker-related subfamily, beta member 3	Hs01085073_m1	Potassium (K [⁺]) channel	
	CNB1	Kv2.1	potassium voltage-gated channel, Shab-related subfamily, member 1	Hs00270657_m1	Potassium (K ⁺) channel	
ŀ	CNC4	Kv3.4	potassium voltage-gated channel, Shaw-related subfamily, member 4		Potassium (K ⁺) channel	
6	CND2	Kv4.2	potassium voltage-gated channel, Shal-related subfamily. member 7	Hs01054873 m1	Potassium (K ⁺) channel	
	CND3	Kv4.3	potassium voltage-gated channel. Shal-related subfamily, member 3	Hs00542597 m1	Potassium (K ⁺) channel	
	CNF1	MinK	notassium voltage-gated channel isk-related family member 1	Hs00264799 s1	Potassium (K ⁺) channel	
Ľ	CNE1	MIRDA	potassium voltage gated channel, isk related family, member 1 like	H:01085745 c1	Potassium (K ⁺) shannel	
Ľ			potassium voltage-gated channel, isk-related family, member 1-like	1501083745_51	Potassium (K ⁺) channel	
1		MIRPI	potassium voltage-gated channel, isk-related family, member 2	H500270822_S1	Potassium (K ⁺) channel	
Ľ	CINES	IVIIRP2	potassium voltage-gated channel, isk-related family, member 3	HS01921343_S1	Potassium (K) channel	
ľ	CNE4	MIRP3	potassium voltage-gated channel, isk-related family, member 4	HS01851577_S1	Potassium (K.) channel	
ľ	CNHZ	NEKG	potassium voltage-gated channel, subtamily H (eag-related), member 2	HS04234270_g1	Potassium (K.) channel	
	CNIPZ	KChIP2	Kv channel interacting protein 2	Hs01552688_g1	Potassium (K [*]) channel	
	CNJ11	Kir6.2	potassium inwardly-rectifying channel, subfamily J, member 11	Hs00265026_s1	Potassium (K') channel	
P	CNJ2	Kir2.1	potassium inwardly-rectifying channel, subfamily J, member 2	Hs01876357_s1	Potassium (K [*]) channel	
P	CNJ3	Kir3.1	potassium inwardly-rectifying channel, subfamily J, member 3	Hs04334861_s1	Potassium (K ⁺) channel	
P	CNJ4	Kir2.3	potassium inwardly-rectifying channel, subfamily J, member 4	Hs00705379_s1	Potassium (K ⁺) channel	
	CNJ5	Kir3.4	potassium inwardly-rectifying channel, subfamily J, member 5	Hs00168476_m1	Potassium (K ⁺) channel	
Þ	CNJ8	Kir6.1	potassium inwardly-rectifying channel, subfamily J, member 8	Hs00958961_m1	Potassium (K ⁺) channel	
Þ	CNK1	TWIK1	potassium channel, subfamily K, member 1	Hs00158428_m1	Potassium (K ⁺) channel	
P	CNK3	TASK1	potassium channel, subfamily K, member 3	Hs00605529_m1	Potassium (K ⁺) channel	
	CNK5	TASK2	potassium channel, subfamily K, member 5	Hs00186652 m1	Potassium (K ⁺) channel	
	CNO1	KvLOT1	potassium voltage-gated channel. KOT-like subfamily, member 1	Hs00923522 m1	Potassium (K ⁺) channel	
r	IPPA	ANP	natriuretic peptide precursor A	Hs00383230 g1	Signaling molecule	
	IPPB	BNP	natriuretic peptide precursor B	Hs01057466 g1	Signaling molecule	
	1N	PIN	nhospholamban	Hs01848144_s1	Calcium (Ca ⁺⁺) regulator	
Ľ.	DD3CA	CAM-DRD	protein phosphatase 3 (formerly 28) catalytic subunit alpha isoform	Hc00174223 m1	Calcium (Ca ⁺⁺) regulator	
Ľ	VP2	EVE2	protein phosphatase 5 (formeny 26), catalytic subanit, alpha isoform	H:00181461 m1	Calcium (Ca ⁺⁺) regulator	
Ľ	CN10A	Novel 9	radium channel veltage gated tune X alpha	Hc01045127 m1	Cadium (Na ⁺) channel	
	CNIDA	Navi.o	source channel, voltage-gated, type X, alpha	1501043137_111	Sodium (Na ⁺) channel	
2	CNID	Navoi	sodium channel, voltage-gated, type I, beta	H503987893_III1	Sodium (Na) channel	
S		NavUZ	sourum channel, voltage-gated, type II, Deta	1500394952_111	Soulum (Iva.) channel	
S	CN3A	INAV1.5	sourum channer, vortage-gated, type III, alpha	ns00306913_m1	sodium (Na.) channel	
S	CN3B	Navb3	socium channel, voltage-gated, type III, beta	HSU1024483_m1	sodium (Na') channel	
S	CN4A	Nav1.4	sodium channel, voltage-gated, type IV, alpha	Hs01109480_m1	Sodium (Na') channel	
S	CN4B	Navb4	sodium channel, voltage-gated, type IV, beta	Hs03681025_m1	Sodium (Na [*]) channel	
S	CN5A	Nav1.5	sodium channel, voltage-gated, type V, alpha (long QT syndrome 3)	Hs00165693_m1	Sodium (Na ⁺) channel	
s	CN7A	Nav2.1	sodium channel, voltage-gated, type VII, alpha	Hs00161546_m1	Sodium (Na ⁺) channel	
s	CN9A	Nav1.7	sodium channel, voltage-gated, type IX, alpha	Hs00161567_m1	Sodium (Na [*]) channel	
s	LC8A1	NCX1	solute carrier family 8 (sodium/calcium exchanger), member 1	Hs01062258_m1	Calcium (Ca ⁺⁺) regulator	
ļ	NK2	ANKB	ankyrin 2	Hs00153998_m1	Cytoskeletal protein	
¢	GATA3	GATA3	GATA Binding Protein 3	Hs00231122_m1	Transcription factor	
¢	ATA4	GATA4	GATA Binding Protein 4	Hs00171403_m1	Transcription factor	
d	GATA5	GATA5	GATA Binding Protein 5	Hs00388359_m1	Transcription factor	
6	ATA6	GATA6	GATA Binding Protein 6	- Hs00232018 m1	Transcription factor	
- J.	IEY2	HEY2	Hes Related Family BHLH Transcription Factor With YRPW Motif 2	Hs00232622 m1	Transcription factor	
- Ji	RX3	IRX3	iroquois homeobox 3	Hs01124217 g1	Transcription factor	
- fi	RXA	IRX4	iroquois homeobox 4	Hs00212560 m ¹	Transcription factor	
Ľ	825	IRX5	iroquois homeobox 5	Hs04334740 m1	Transcription factor	
Ľ	BX7	TBX2	T-Box Transcription Factor 2	Hs00911920 m1	Transcription factor	
Ľ	DV2	TRVO	T Pay Transcription Factor 2	Hc00105612 m1	Transcription actor	
Ľ			T Pox Transcription Factor 5	H-003611EL1	Transcription factor	
ľ			N/2 Hamaahan E	ms00301125_M1	manscription factor	
r	IKX2.5	NKX2-5	NKZ HOMEODOX 5	HSUU231763_m1	ranscription factor	
1	NNI3	TNNI3	Troponin 13, Cardiac Type	Hs00165957_m1	Cytoskeletal protein	
ľ	NNT2	TNNT2	Troponin T2, Cardiac Type	Hs00943911_m1	Cytoskeletal protein	
ľ	/IYH6	MYH6	Myosin Heavy Chain 6	Hs01101425_m1	Cytoskeletal protein	
r	/YH7	MYH7	Myosin Heavy Chain 7	Hs01110632_m1	Cytoskeletal protein	
F	RAD	RAD	RRAD, Ras Related Glycolysis Inhibitor And Calcium Channel Regulator	Hs00188163_m1	GTP-binding protein	
c	LASP2	CLASP2	Cytoplasmic Linker Associated Protein 2	Hs00380556_m1	Cytoskeletal protein	
c	PD1L	GPD1L	Glycerol-3-Phosphate Dehydrogenase 1 Like	Hs00380518_m1	Enzyme	
	/IYL7	MLC2A	Myosin Light Chain 7	Hs01085598_g1	Cytoskeletal protein	
		MICOV	Mvosin Light Chain 2	Hs00166405_m1	Cytoskeletal protein	
r	AYL2	IVILCZV		_		
r 1	ЛҮL2 85	IVILC2 V	Eukaryotic 18s rRNA	Hs99999901 s1	endogenous control	
P P 1	/IYL2 85 32M	B2M	Eukaryotic 18s rRNA Beta-2-Mirroglobulin	Hs999999901_s1 Hs00187842_m1	endogenous control	
r 1 1	AYL2 85 52M ACTB	B2M ACTB	Eukaryotic 18s rRNA Beta-2-Microglobulin actin beta	Hs99999901_s1 Hs00187842_m1 Hs99999903_m1	endogenous control endogenous control endogenous control	

Table S4. Mean value \pm SEM of I_{Na} and $I_{Ca,L}$ activation and inactivation kinetics parameters in the different hiPSC-CMs lines. V1/2 and K represent voltage of half-maximum (in)activation and slope factor, respectively.

		8	Ctrl	BrS1 ⁺	BrS2 ⁺	BrS3-	BrS4 ⁻	BrS5	BrS6	Non- BrS
	Activation	V _{1/2}	-33.22	-32.54	-33.55	-32.91	-33.79	-33.58	-33.22	-33.21
		(mV)	± 0.3	± 0.2	± 0.6	± 0.2	± 0.5	± 0.2	± 0.6	± 0.2
		K	6.244	6.122	6.553	6.369	6.372	5.689	6.935	5.947
т		(mV)	± 0.2	± 0.2	± 0.4	± 0.1	± 0.4	± 0.1	± 0.4	± 0.1
I _{Na}	Inactivation	V _{1/2}	-87.17	-86.4	-88.13	-85.51	-87.53	-85.91	-86.92	-88.12
		(mV)	± 0.2	± 0.4	± 0.6	± 0.2	± 0.3	± 0.2	± 0.2	± 0.4
		K	-5.691	-5.448	-6.381	-6.262	-6.029	-6.004	-5.609	-5.444
		(mV)	± 0.1	± 0.3	± 0.5	± 0.2	± 0.2	± 0.1	± 0.1	± 0.4
	Activation	$V_{1/2}$	-16.62	-18.47	-1 9.41	-17.79	-17.21	-18.54	-19.71	-18.49
		(mV)	± 0.3	± 0.3	± 1.1	± 0.3	± 1.1	± 0.3	± 0.4	± 0.6
		K	7.006	6.064	7.522	6.86	6.777	6.265	6.322	6.68
-		(mV)	± 0.2	± 0.2	± 0.8	± 0.2	± 0.8	± 0.2	± 0.3	± 0.4
I _{Ca,L}	Inactivation	V _{1/2}	-46.92	-44.85	-49.38	-47.49	-49.69	-49.69	-44.8	-46.1
		(mV)	± 0.2	± 0.2	± 0.5	± 0.2	± 0.5	± 0.2	± 0.4	± 0.3
		K	-7.429	- 6.406	-6.843	-7.251	-8.087	-7.167	-7.257	-6.581
		(mV)	± 0.2	± 0.2	± 0.4	± 0.2	± 0.4	± 0.2	± 0.4	± 0.3

Table S5. Correlation between ECG parameters and the corresponding hiPSC-CM sodium currents.

		Correlation coefficient r_s of Spearman		
		Late I _{Na}	Peak I _{Na}	
	RR (ms)	0.48	-0.07	
D1	S duration (ms)	-0.55	0.00	
	S amplitude (mm)	-0.10	0.26	
	P (ms)	-0.47	0.01	
	PR (ms)	-0.63	0.20	
D2	QRS (ms)	-0.55	-0.31	
	QTp (ms)	-0.40	-0.14	
	QTe (ms)	-0.32	-0.14	
	TPE (ms)	-0.07	-0.14	
V1	J amplitude (mm)	-0.81 *	0.02	
	TPE (ms)	0.11	-0.14	
V2	J amplitude (mm)	-0.86 *	0.14	
	TPE (ms)	-0.33	-0.26	
V3	J amplitude (mm)	0.23	0.19	
	TPE (ms)	-0.23	-0.31	

Each ECG parameter was tested for its correlation with either $I_{Na,L}$ or peak I_{Na} density from the corresponding hiPSC-CMs. While the J point elevation correlated significantly with $I_{Na,L}$ density, it did not correlate with hiPSC-CM peak I_{Na} density. Statistical significance threshold of r_s was p < 0.05 (*).